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NF 38

THE NATIONAL FORMULARY

Volume 5

By authority of the United States Pharmacopeial Convention.
Prepared by the Council of Experts and its Expert Committees

Official from May 1, 2020

The designation on the cover of this publication, "USP NF 2020," is for ease of identification only. The publication contains two separate compendia: The United States Pharmacopeia, Forty-Third Revision, and The National Formulary, Thirty-Eighth Edition.

THE UNITED STATES PHARMACOPEIAL CONVENTION
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SIX-MONTH IMPLEMENTATION GUIDELINE

The *United States Pharmacopeia–National Formulary* and its supplements become official **six months** after being released to the public. The *USP–NF,* which is released on November 1 of each year, becomes official on May 1 of the following year. This six-month implementation timing gives users more time to bring their methods and procedures into compliance with new and revised *USP–NF* requirements.

The table below describes the official dates of the *USP–NF* and its supplements. The 2019 *USP 42–NF 37,* and its supplements, *Interim Revision Announcements (IRAs)* and *Revision Bulletins* to that edition, will be official until May 1, 2020, at which time the *USP 43–NF 38* becomes official.

Publication	Release Date	Official Date	Official Until
USP 43–NF 38	November 1, 2019	May 1, 2020	May 1, 2021 (except as superseded by supplements, *IRAs,* and *Revision Bulletins*)
First Supplement to the USP 43–NF 38	February 1, 2020	August 1, 2020	May 1, 2021 (except as superseded by *Second Supplement, IRAs,* and *Revision Bulletins*)
Second Supplement to the USP 43–NF 38	June 1, 2020	December 1, 2020	May 1, 2021 (except as superseded by *IRAs* and *Revision Bulletins*)
USP-NF 2021 Issue 1	November 1, 2020	May 1, 2021	May 1, 2022 (except as superseded by supplements, *IRAs,* and *Revision Bulletins*)

The table below gives the details of the *IRAs* that will apply to *USP 43–NF 38.*

IRA	PF Posting Date	Comment Due Date	IRA Posting Date	IRA Official Date
46(1)	January 2, 2020	March 31, 2020	May 29, 2020	July 1, 2020
46(2)	March 2, 2020	May 31, 2020	July 31, 2020	September 1, 2020
46(3)	May 1, 2020	July 31, 2020	September 25, 2020	November 1, 2020
46(4)	July 1, 2020	September 30, 2020	November 20, 2020	January 1, 2021
46(5)	September 1, 2020	November 30, 2020	January 31, 2021	March 1, 2021
46(6)	November 2, 2020	January 31, 2020	March 27, 2021	May 1, 2021

Revision Bulletins are published on the USP website and the *USP–NF Online* product and become official on the date specified in the *Revision Bulletin.*

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GENERAL NOTICES AND REQUIREMENTS

The *General Notices and Requirements* section (the *General Notices*) presents the basic assumptions, definitions, and default conditions for the interpretation and application of the *United States Pharmacopeia* (*USP*) and the *National Formulary* (*NF*).

Requirements stated in these *General Notices* apply to all articles recognized in the *USP* and *NF* (the "compendia") and to all general chapters unless specifically stated otherwise.

1. TITLE AND REVISION

The full title of this publication (consisting of five volumes and including its *Supplements*), is *The Pharmacopeia of the United States of America*, Forty-Third Revision and the *National Formulary*, Thirty-Eighth Edition. These titles may be abbreviated to *USP 43*, to *NF 38*, and to *USP 43–NF 38*. The *United States Pharmacopeia*, Forty-Third Revision, and the *National Formulary*, Thirty-Eighth Edition, supersede all earlier revisions. Where the terms "*USP*," "*NF*," or "*USP–NF*" are used without further qualification during the period in which these compendia are official, they refer only to *USP 43*, *NF 38*, and any *Supplement(s)* thereto. The same titles, with no further distinction, apply equally to print or electronic presentation of these contents. Although *USP* and *NF* are published under one cover and share these *General Notices*, they are separate compendia.

This revision is official beginning May 1, 2020 unless otherwise indicated in specific text.

Supplements to *USP* and *NF* are published periodically.

Accelerated Revisions, published periodically on the *Official Text* section of USP's website (http://www.usp.org/usp-nf/official-text), are designed to make revisions official more quickly than through the routine process for publishing standards in the *USP–NF*. *Interim Revision Announcements* are Accelerated Revisions to *USP* and *NF* that contain official revisions and their effective dates.

Revision Bulletins are Accelerated Revisions to official text or postponements that require expedited publication. They generally are official immediately unless otherwise specified in the *Revision Bulletin*.

Errata are Accelerated Revisions representing corrections to items erroneously published. Announcements of the availability of new USP Reference Standards and announcements of tests or procedures that are held in abeyance pending availability of required USP Reference Standards are also available on the "Official Text" tab of USP's website.

2. OFFICIAL STATUS AND LEGAL RECOGNITION

2.10. Official Text

Official text of the *USP* and *NF* is published in the *USP–NF Online* (www.uspnf.com) in the edition identified as "CURRENTLY OFFICIAL" and in Accelerated Revisions that supersede the *USP–NF Online* as described below.

Routine revisions are published in the *USP–NF Online* and become official on the date indicated, usually six months after publication. Accelerated Revisions supersede the *USP–NF Online* and become official on the date indicated. Links to Accelerated Revisions on the USP website can be found in any superseded monograph or general chapter in the *USP–NF Online*.

Print and USB flash drive versions of the *USP* and *NF* also are available. Routine revisions are provided with the same timing as the *USP–NF Online*. Official text published in *Supplements* supersedes that in the previously published print or USB flash drive versions of *USP–NF*. These versions also are superseded by Accelerated Revisions as described above.

In the event of any disparity between the print or USB flash drive versions and the *USP–NF Online*, the *USP–NF Online* will be deemed to apply.

2.20. Official Articles

An *official article* is an article that is recognized in *USP* or *NF*. An article is deemed to be recognized and included in a compendium when a monograph for the article is published in the compendium and an official date is generally or specifically assigned to the monograph.

The title specified in a monograph is the *official title* for such article. Other names considered to be synonyms of the *official titles* may not be used as substitutes for *official titles*. For drug products that incorporate a sensor to detect that the product has been administered, the *official title* shall be the title specified in the relevant drug product monograph plus the words "with sensor".

Official articles include both *official substances* and *official products*. An *official substance* is a drug substance, excipient, dietary ingredient, other ingredient, or component of a finished device for which the monograph title includes no indication of the nature of the finished form.

An *official product* is a drug product, dietary supplement, compounded preparation, or finished device for which a monograph is provided.

2.30. Legal Recognition

The *USP* and *NF* are recognized in the laws and regulations of many countries throughout the world. Regulatory authorities may enforce the standards presented in the *USP* and *NF*, but because recognition of the *USP* and *NF* may vary by country, users should understand applicable laws and regulations. In the United States under the Federal Food, Drug, and Cosmetic Act (FDCA), both *USP* and *NF* are recognized as official compendia. A drug with a name recognized in *USP–NF* must comply with compendial identity standards or be deemed adulterated, misbranded, or both. See, e.g., FDCA § 501(b) and 502(e)(3)(b); also FDA regulations, 21 CFR § 299.5(a&b). To avoid being deemed adulterated, such drugs must also comply with compendial standards for strength, quality, and purity, unless labeled to show all respects in which the drug differs. See, e.g., FDCA § 501(b) and 21 CFR § 299.5(c). In addition, to avoid being deemed misbranded, drugs recognized in *USP–NF* must also be packaged and

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labeled in compliance with compendial standards. See FDCA § 502(g).

A dietary supplement represented as conforming to specifications in *USP* will be deemed a misbranded food if it fails to so conform. See FDCA § 403(s)(2)(D).

Enforcement of *USP* standards is the responsibility of FDA and other government authorities in the U.S. and elsewhere. USP has no role in enforcement.

3. CONFORMANCE TO STANDARDS

3.10. Applicability of Standards

Standards for an article recognized in the compendia (*USP–NF*) are expressed in the article's monograph, applicable general chapters, and *General Notices*. The identity, strength, quality, and purity of an article are determined by the official tests, procedures, and acceptance criteria, and other requirements incorporated in the monograph, in applicable general chapters, or in the *General Notices*. "Applicable general chapters" means general chapters numbered below 1000 or above 2000 that are made applicable to an article through reference in *General Notices*, a monograph, or another applicable general chapter numbered below 1000. Where the requirements of a monograph differ from the requirements specified in these *General Notices* or an applicable general chapter, the monograph requirements apply and supersede the requirements of the *General Notices* or applicable general chapters, whether or not the monograph explicitly states the difference.

General chapters numbered 1000 to 1999 are for informational purposes only. They contain no mandatory tests, assays, or other requirements applicable to any official article, regardless of citation in a general chapter numbered below 1000, a monograph, or these *General Notices*. General chapters numbered above 2000 apply only to articles that are intended for use as dietary ingredients and dietary supplements. General chapter citations in *NF* monographs refer to *USP* general chapters.

Early adoption of revised standards in advance of the official date is allowed by USP unless specified otherwise at the time of publication. Where revised standards for an existing article have been published as final approved "official text" (as approved in section *2.10 Official Text*) but have not yet reached the official date (6 months after publication, unless otherwise specified; see "official date", section *2.20 Official Articles*), compliance with the revised standard shall not preclude a finding or indication of conformance with compendial standards, unless USP specifies otherwise by prohibiting early adoption in a particular standard.

The standards in the relevant monograph, general chapter(s), and *General Notices* apply at all times in the life of the article from production to expiration. It is also noted that the manufacturer's specifications, and manufacturing practices (e.g., Quality by Design, Process Analytical Technology, and Real Time Release Testing initiatives), generally are followed to ensure that the article will comply with compendial standards until its expiration date, when stored as directed. Every compendial article in commerce shall be so constituted that when examined in accordance with these assays and test procedures, it meets all applicable pharmacopeial requirements (*General Notices*, monographs, and general chapters). Thus, any official article is expected to meet the compendial standards if tested, and any official article actually tested as directed in the relevant monograph must meet such standards to demonstrate compliance.

Some tests, such as those for *Dissolution* and *Uniformity of Dosage Units*, require multiple dosage units in conjunction with a decision scheme. These tests, albeit using a number of dosage units, are in fact one determination. These procedures should not be confused with statistical sampling plans. The similarity to statistical procedures may seem to suggest an intent to make inference to some larger group of units, but in all cases, statements about whether the compendial standard is met apply only to the units tested. Repeats, replicates, statistical rejection of outliers, or extrapolations of results to larger populations, as well as the necessity and appropriate frequency of batch testing, are neither specified nor proscribed by the compendia; such decisions are based on the objectives of the testing. Frequency of testing and sampling are left to the preferences or direction of those performing compliance testing, and other users of *USP–NF*, including manufacturers, buyers, or regulatory authorities.

Official products are prepared according to recognized principles of good manufacturing practice and from ingredients that meet *USP* or *NF* standards, where standards for such ingredients exist (for dietary supplements, see section *3.10.20 Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients*).

Official substances are prepared according to recognized principles of good manufacturing practice and from ingredients complying with specifications designed to ensure that the resultant substances meet the requirements of the compendial monographs.

3.10.10. Applicability of Standards to Drug Products, Drug Substances, and Excipients

The applicable *USP* or *NF* standard applies to any article marketed in the United States that (1) is recognized in the compendium and (2) is intended or labeled for use as a drug or as an ingredient in a drug. Such articles (drug products, drug substances, and excipients) include both human drugs (whether dispensed by prescription, "over the counter," or otherwise), as well as animal drugs. The applicable standard applies to such articles whether or not the added designation "USP" or "NF" is used. The standards apply equally to articles bearing the official titles or names derived by transposition of the definitive words of official titles or transposition in the order of the names of two or more drug substances in official titles, or where there is use of synonyms with the intent or effect of suggesting a significant degree of identity with the official title or name.

3.10.20. Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients

An article recognized in *USP* or *NF* shall comply with the compendial standards if the article is a medical device, component intended for a medical device, dietary supplement, dietary ingredient, or other ingredient that is intended for incorporation into a dietary supplement, and is labeled as conforming to the *USP* or *NF*.

Generally, dietary supplements are prepared from ingredients that meet *USP*, *NF*, or *Food Chemicals Codex* standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to be of acceptable food grade quality using other suitable procedures.

3.10.30. Applicability of Standards to the Practice of Compounding

USP compounding practice standards, *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, as appropriate, apply to compounding practice or activity regardless of whether a monograph exists for the compounded preparation or these chapters are referenced in such a monograph. In the United States, ⟨795⟩ and ⟨797⟩ are not applicable to drugs compounded by entities registered with FDA as outsourcing facilities as defined by FDCA § 503B, because such facilities are required to comply with FDA's current good manufacturing practice requirements. Compounded preparations, including drug products compounded by outsourcing facilities, may also be

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subject to applicable monographs; see section *2.20 Official Articles* and section *4.10 Monographs*.

3.20. Indicating Conformance

A drug product, drug substance, or excipient may use the designation "USP" or "NF" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the identity prescribed in the specified compendium.

When a drug product, drug substance, compounded preparation, or excipient differs from the relevant *USP* or *NF* standard of strength, quality, or purity, as determined by the application of the tests, procedures, and acceptance criteria set forth in the relevant compendium, its difference shall be plainly stated on its label.

When a drug product, drug substance, compounded preparation, or excipient fails to comply with the identity prescribed in *USP* or *NF* or contains an added substance that interferes with the prescribed tests and procedures, the article shall be designated by a name that is clearly distinguishing and differentiating from any name recognized in *USP* or *NF*.

A medical device, dietary supplement, or ingredient or component of a medical device or dietary supplement may use the designation "USP" or "NF" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the monograph standards and other applicable standards in that compendium.

The designation "USP" or "NF" on the label may not and does not constitute an endorsement by USP and does not represent assurance by USP that the article is known to comply with the relevant standards. USP may seek legal redress if an article purports to be or is represented as an official article in one of USP's compendia and such claim is determined by USP not to be made in good faith.

The designation "USP–NF" may be used on the label of an article provided that the label also bears a statement such as "Meets *NF* standards as published by USP," indicating the particular compendium to which the article purports to apply.

When the letters "USP," "NF," or "USP–NF" are used on the label of an article to indicate compliance with compendial standards, the letters shall appear in conjunction with the official title of the article. The letters are not to be enclosed in any symbol such as a circle, square, etc., and shall appear in capital letters.

If a dietary supplement does not comply with all applicable compendial requirements but contains one or more dietary ingredients or other ingredients that are recognized in *USP* or *NF*, the individual ingredient(s) may be designated as complying with *USP* or *NF* standards or being of *USP* or *NF* quality provided that the designation is limited to the individual ingredient(s) and does not suggest that the dietary supplement complies with *USP* standards.

4. MONOGRAPHS AND GENERAL CHAPTERS

4.10. Monographs

Monographs set forth the article's name, definition, specification, and other requirements related to packaging, storage, and labeling. The specification consists of tests, procedures, and acceptance criteria that help ensure the identity, strength, quality, and purity of the article. For general requirements relating to specific monograph sections, see section *5. Monograph Components*.

Because monographs may not provide standards for all relevant characteristics, some official substances may conform to the *USP* or *NF* standard but differ with regard to nonstandardized properties that are relevant to their use in specific preparations. To assure substitutability in such instances, users may wish to ascertain functional equivalence or determine such characteristics before use.

4.10.10. Applicability of Test Procedures

A single monograph may include more than one test, procedure, and/or acceptance criterion for the same attribute. Unless otherwise specified in the monograph, all tests are requirements. In some cases, monograph instructions allow the selection of tests that reflect attributes of different manufacturers' articles, such as different polymorphic forms, impurities, hydrates, and dissolution. Monograph instructions indicate the tests, procedures, and/or acceptance criteria to be used and the required labeling.

The order in which the tests are listed in the monograph is based on the order in which they are approved by the relevant Expert Committee for inclusion in the monograph. Test 1 is not necessarily the test for the innovator or for the reference product. Depending on monograph instructions, a labeling statement is not typically required if Test 1 is used.

4.10.20. Acceptance Criteria

The acceptance criteria allow for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions. The existence of compendial acceptance criteria does not constitute a basis for a claim that an official substance that more nearly approaches 100% purity "exceeds" compendial quality. Similarly, the fact that an article has been prepared to tighter criteria than those specified in the monograph does not constitute a basis for a claim that the article "exceeds" the compendial requirements.

An official product shall be formulated with the intent to provide 100% of the quantity of each ingredient declared on the label. Where the minimum amount of a substance present in a dietary supplement is required by law to be higher than the lower acceptance criterion allowed for in the monograph, the upper acceptance criterion contained in the monograph may be increased by a corresponding amount.

The acceptance criteria specified in individual monographs and in the general chapters for compounded preparations are based on such attributes of quality as might be expected to characterize an article compounded from suitable bulk drug substances and ingredients, using the procedures provided or recognized principles of good compounding practice, as described in these compendia.

4.20. General Chapters

Each general chapter is assigned a number that appears in angle brackets adjacent to the chapter name (e.g., *Chromatography* ⟨621⟩). General chapters may contain the following:

- Descriptions of tests and procedures for application through individual monographs,
- Descriptions and specifications of conditions and practices for pharmaceutical compounding,
- General information for the interpretation of the compendial requirements,
- Descriptions of general pharmaceutical storage, dispensing, and packaging practices, or
- General guidance to manufacturers of official substances or official products.

When a general chapter is referenced in a monograph, acceptance criteria may be presented after a colon.

Some chapters may serve as introductory overviews of a test or of analytical techniques. They may reference other general chapters that contain techniques, details of the procedures, and, at times, acceptance criteria.

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5. MONOGRAPH COMPONENTS

5.10. Molecular Formula

The use of the molecular formula for the official substance(s) named in defining the required strength of a compendial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100%) purity.

5.20. Added Substances

Added substances are presumed to be unsuitable for inclusion in an official article and therefore prohibited, if their presence impairs the bioavailability, therapeutic efficacy, or safety of the official article; or they interfere with the assays and tests prescribed for determining compliance with the compendial standards (see section *3.20 Indicating Conformance*).

The air in a container of an official article may, where appropriate, be evacuated or be replaced by carbon dioxide, helium, argon, or nitrogen, or by a mixture of these gases. The use of such gas need not be declared in the labeling.

5.20.10. Added Substances in Official Substances

Official substances may contain only the specific added substances that are permitted by the individual monograph. Such added substances shall not exceed the quantity required for providing their intended effect. Where such addition is permitted, the label shall indicate the name(s) and amount(s) of any added substance(s).

5.20.20. Added Substances (Excipients and Ingredients) in Official Products

Suitable substances and excipients such as antimicrobial agents, pharmaceutical bases, carriers, coatings, flavors, preservatives, stabilizers, and vehicles may be added to an official product to enhance its stability, usefulness, or elegance, or to facilitate its preparation, unless otherwise specified in the individual monograph.

Added substances and excipients employed solely to impart color may be incorporated into official products other than those intended for parenteral or ophthalmic use, in accordance with the regulations pertaining to the use of colors issued by the FDA, provided such added substances or excipients are otherwise appropriate in all respects. (See also *Injections and Implanted Drugs Products* ⟨1⟩, *Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and added substances, Added substances*.)

The proportions of the substances constituting the base in ointment and suppository products and preparations may be varied to maintain a suitable consistency under different climatic conditions, provided that the concentrations of drug substances are not varied and provided that the bioavailability, therapeutic efficacy, and safety of the preparation are not impaired.

5.20.20.1. In Compounded Preparations

Compounded preparations for which a complete composition is given shall contain only the ingredients named in the formulas unless specifically exempted herein or in the individual monograph. Deviation from the specified processes or methods of compounding, although not from the ingredients or proportions thereof, may occur provided that the finished preparation conforms to the relevant standards and to preparations produced by following the specified process.

Where a monograph for a compounded preparation calls for an ingredient in an amount expressed on the dried basis, the ingredient need not be dried before use if due allowance is made for the water or other volatile substances present in the quantity taken.

Specially denatured alcohol formulas are available for use in accordance with federal statutes and regulations of the Internal Revenue Service. A suitable formula of specially denatured alcohol may be substituted for Alcohol in the manufacture of official preparations intended for internal or topical use, provided that the denaturant is volatile and does not remain in the finished product. A preparation that is intended for topical application to the skin may contain specially denatured alcohol, provided that the denaturant is either a usual ingredient in the preparation or a permissible added substance; in either case the denaturant shall be identified on the label of the topical preparation. Where a process is given in the individual monograph, any preparation compounded using denatured alcohol shall be identical to that prepared by the monograph process.

5.20.20.2. In Dietary Supplements

Additional ingredients may be added to dietary supplement products provided that the additional ingredients (1) comply with applicable regulatory requirements, and (2) do not interfere with the assays and tests prescribed for determining compliance with compendial standards.

5.30. Description and Solubility

Only where a quantitative solubility test is given in a monograph and is designated as such is it a test for purity.

A monograph may include information regarding the article's description. Information about an article's "description and solubility" also is provided in the reference table *Description and Relative Solubility of USP and NF Articles*. The reference table merely denotes the properties of articles that comply with monograph standards. The reference table is intended primarily for those who use, prepare, and dispense drugs and/or related articles. Although the information provided in monographs and the information in the reference table may indirectly assist in the preliminary evaluation of an article, it is not intended to serve as a standard or test for purity.

The approximate solubility of a compendial substance is indicated by one of the following descriptive terms:

Descriptive Term	Parts of Solvent Required for 1 Part of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1,000
Very slightly soluble	From 1,000 to 10,000
Practically insoluble, or Insoluble	Greater than or equal to 10,000

5.40. Identification

A compendial test titled *Identification* is provided as an aid in verifying the identity of articles as they are purported to be, e.g., those taken from labeled containers, and to establish whether it is the article named in *USP–NF*. The *Identification* test for a particular article may consist of one or more procedures. When a compendial *Identification* test is undertaken, all requirements of all specified procedures in the test must be met to satisfy the requirements of the test. Failure of an article to meet all the requirements of a prescribed *Identification* test (i.e., failure to meet the requirements of all of the specified procedures that are components of that test) indicates that the article is mislabeled and/or adulterated.

5.50. Assay

Assay tests for compounded preparations are not intended for evaluating a compounded preparation before dispensing, but instead are intended to serve as the official test in the event of a question or dispute regarding the preparation's conformance to official standards.

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5.50.10. Units of Potency (Biological)

For substances that cannot be completely characterized by chemical or physical means or that need confirmation of functionality or tertiary structure, it may be necessary to express quantities of biological activity in units of biological potency, each defined by an authoritative, designated reference standard. In cases where international reference materials have been discontinued, international units of potency may be defined in terms of molecular mass, such as in the cases of vitamins A, D, and E.

Where available, World Health Organization (WHO) international biological standards define the International Units (IU). *USP* monographs refer to the units assigned by USP Reference Standards either directly as International Units (IU) or as "USP Units." For some biological products, units of potency are value assigned against a corresponding U.S. Standard established by FDA, whether or not International Units or USP Units have been defined (see *Biologics* ⟨1041⟩). Note that product-related labeling, e.g., on containers, need not use the full phrase "USP [product name] Units" that appears in many *USP* monograph labeling sections. The term "USP Units" can be used on product labeling consistent with USP compendial requirements, provided it is clear from the context that the potency is stated in terms of USP [product name] Units. In such circumstances it should be clear that "USP Units" and "USP [product name] Units" share the same meaning.

5.60. Impurities and Foreign Substances

Tests for the presence of impurities and foreign substances are provided to limit such substances to amounts that are unobjectionable under conditions in which the article is customarily employed (see also *Impurities in Drug Substances and Drug Products* ⟨1086⟩).

Nonmonograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practices.

5.60.10. Other Impurities in USP and NF Articles

If a *USP* or *NF* monograph includes an assay or organic impurity test based on chromatography, other than a test for residual solvents, and that monograph procedure does not detect an impurity present in the substance, the amount and identity of the impurity, where both are known, shall be stated in the labeling (certificate of analysis) of the official substance, under the heading *Other Impurity(ies)*.

The presence of any unlabeled other impurity in an official substance is a variance from the standard if the content is 0.1% or greater. The sum of all *Other Impurities* combined with the monograph-detected impurities may not exceed 2.0% (see *Ordinary Impurities* ⟨466⟩), unless otherwise stated in the monograph.

The following categories of drug substances are excluded from *Other Impurities* requirements:

- Fermentation products and semi-synthetics derived therefrom,
- Radiopharmaceuticals,
- Biologics,
- Biotechnology-derived products,
- Peptides,
- Herbals, and
- Crude products of animal or plant origin.

Any substance known to be toxic shall not be listed under *Other Impurities*.

5.60.20. Residual Solvents in USP and NF Articles

All *USP* and *NF* articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration, and the solvents limited according to the principles defined and the requirements specified in *Residual Solvents* ⟨467⟩, using the general methods presented therein or other suitable methods.

5.60.30. Elemental Impurities in USP Drug Products and Dietary Supplements

Elemental impurities in official drug products are controlled according to the principles defined and requirements specified in *Elemental Impurities—Limits* ⟨232⟩. Elemental contaminants in official dietary supplements are controlled according to the principles defined and requirements specified in *Elemental Contaminants in Dietary Supplements* ⟨2232⟩.

5.70. Performance Tests

Where content uniformity determinations have been made using the same analytical methodology specified in the *Assay*, with appropriate allowances made for differences in sample preparation, the average of all of the individual content uniformity determinations may be used as the *Assay* value.

5.80. USP Reference Standards

USP Reference Standards are authentic specimens that have been approved as suitable for use as comparison standards in *USP* or *NF* tests and assays. (See *USP Reference Standards* ⟨11⟩.) Where *USP* or *NF* tests or assays call for the use of a USP Reference Standard, only those results obtained using the specified USP Reference Standard are conclusive. Where a procedure calls for the use of a compendial article rather than for a USP Reference Standard as a material standard of reference, a substance meeting all of the compendial monograph requirements for that article shall be used. If any new *USP* or *NF* standard requires the use of a new USP Reference Standard that is not yet available, that portion of the standard containing the requirement shall not be official until the specified USP reference material is available.

Unless a Reference Standard label bears a specific potency or content, assume the Reference Standard is 100.0% pure in the official application. Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP Reference Standards are to be used in accordance with the instructions on the label of the Reference Standard.

6. TESTING PRACTICES AND PROCEDURES

6.10. Safe Laboratory Practices

In performing compendial procedures, safe laboratory practices shall be followed, including precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any procedure described in the compendia, the analyst should be aware of the hazards associated with the chemicals and the techniques and means of protecting against them. These compendia are not designed to describe such hazards or protective measures.

6.20. Automated Procedures

Automated and manual procedures employing the same basic chemistry are considered equivalent provided the automated system is properly qualified as being suitable to execute the compendial manual method and the analytical procedure is verified under the new equipment conditions.

6.30. Alternative and Harmonized Methods and Procedures

An alternative method or procedure is defined as any method or procedure other than the compendial method or

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procedure for the article in question. The alternative method or procedure must be fully validated (see *Validation of Compendial Procedures* ⟨1225⟩) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis. Alternative methods or procedures can be developed for any one of a number of reasons not limited to simplification of sample preparation, enhanced precision and accuracy, improved (shortened) run time, or being better suited to automation than the compendial method or procedure. Only those results obtained by the methods and procedures given in the compendia are conclusive.

For evaluation as a potential replacement or addition to the standard, alternative methods and procedures should be submitted to USP (see section *4.10 Monographs*).

Certain general chapters contain a statement that the text in question is harmonized with the corresponding text of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* and that these texts are interchangeable. Therefore, if a substance or preparation is found to comply with a requirement using an interchangeable method or procedure from one of these pharmacopeias, it should comply with the requirements of the *USP–NF*. When a difference appears, or in the event of dispute, only the result obtained by the method and/or procedure given in the *USP–NF* is conclusive.

6.40. Dried, Anhydrous, Ignited, or Solvent-Free Basis

All calculations in the compendia assume an "as-is" basis unless otherwise specified.

Test procedures may be performed on the undried or unignited substance and the results calculated on the dried, anhydrous, or ignited basis, provided a test for *Loss on Drying*, or *Water Determination*, or *Loss on Ignition*, respectively, is given in the monograph. Where the presence of moisture or other volatile material may interfere with the procedure, previous drying of the substance is specified in the individual monograph and is obligatory.

The term "solvent-free" signifies that the calculation shall be corrected for the presence of known solvents as determined using the methods described in ⟨467⟩ unless a test for limit of organic solvents is provided in the monograph.

The term "previously dried" without qualification signifies that the substance shall be dried as directed under *Loss on Drying* ⟨731⟩ or *Water Determination* ⟨921⟩ (gravimetric determination).

Where drying in vacuum over a desiccant is directed, a vacuum desiccator, a vacuum drying pistol, or other suitable vacuum drying apparatus shall be used.

6.40.10. Ignite to Constant Weight

"Ignite to constant weight" means that ignition shall be continued at 800 ± 25°, unless otherwise indicated, until two consecutive weighings, the second of which is taken after an additional period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.40.20. Dried to Constant Weight

"Dried to constant weight" means that drying shall be continued until two consecutive weighings, the second of which is taken after an additional drying period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.50. Preparation of Solutions

6.50.10. Filtration

Where a procedure gives direction to "filter" without further qualification, the liquid shall be passed through suitable filter paper or equivalent device until the filtrate is clear. Due to the possibility of filter effects, the initial volumes of a filtrate may be discarded.

6.50.20. Solutions

Unless otherwise specified, all solutions shall be prepared with Purified Water. Solutions for quantitative measures shall be prepared using accurately weighed or accurately measured analytes (see section *8.20 About*).

An expression such as "(1 in 10)" means that 1 part *by volume* of a liquid shall be diluted with, or 1 part *by weight* of a solid shall be dissolved in, a sufficient quantity of the diluent or solvent to make the volume of the finished solution 10 parts *by volume*. For example, a 1 in 10 solution is prepared by diluting 1 mL of a liquid or dissolving 1 g of a solid in sufficient solvent to make 10 mL of the solution. An expression such as "(20:5:2)" means that the respective numbers of parts, by volume, of the designated liquids shall be mixed, unless otherwise indicated.

6.50.20.1. Adjustments to Solutions

When a specified concentration is called for in a procedure, a solution of other normality or molarity may be used, provided that allowance is made for the difference in concentration and that the change does not increase the error of measurement.

Proportionately larger or smaller quantities than the specified weights and volumes of assay or test substances and Reference Standards may be taken, provided the measurement is made with at least equivalent accuracy.

Unless otherwise indicated, analyte concentrations shall be prepared to within ten percent (10%) of the indicated value. In the case in which a procedure is adapted to the working range of an instrument, solution concentrations may differ from the indicated value by more than ten percent (10%), with appropriate changes in associated calculations. Any changes shall fall within the validated range of the instrument.

When adjustment of pH is indicated with either an acid or base and the concentration is not indicated, appropriate concentrations of that acid or base may be used.

6.50.20.2. Test Solutions

Information on Test Solutions (TS) is provided in the *Test Solutions* portion of the *Reagents, Indicators, and Solutions* section of the *USP–NF*. Use of an alternative Test Solution or a change in the Test Solution used may require validation.

6.50.20.3. Indicator Solutions

Where a procedure specifies the use of an indicator TS, approximately 0.2 mL, or 3 drops, of the solution shall be added unless otherwise directed.

6.60. Units Necessary to Complete a Test

Unless otherwise specified, a sufficient number of units to ensure a suitable analytical result shall be taken.

6.60.10. Tablets

Where the procedure of a Tablet monograph directs to weigh and finely powder not fewer than a given number of Tablets, a counted number of Tablets shall be weighed and reduced to a powder. The portion of the powdered Tablets taken shall be representative of the whole Tablets and shall, in turn, be weighed accurately.

6.60.20. Capsules

Where the procedure of a Capsule monograph gives direction to remove, as completely as possible, the contents of not fewer than a given number of the Capsules, a counted number of Capsules shall be carefully opened and the contents quantitatively removed, combined, mixed, and weighed accurately. The portion of mixed Capsules contents taken shall be representative of the contents of the Capsules and shall, in turn, be weighed accurately.

6.70. Reagents

The proper conduct of the compendial procedures and the reliability of the results depend, in part, upon the quality of the reagents used in the performance of the procedures. Unless otherwise specified, reagents conforming to the specifications

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set forth in the current edition of *Reagent Chemicals* published by the American Chemical Society (ACS) shall be used. Where such ACS reagent specifications are not available or where the required purity differs, compendial specifications for reagents of acceptable quality are provided (see the *Reagents, Indicators, and Solutions* section of the *USP–NF*). Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of assay or test involved.

Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to *USP* or *NF* in their labeling shall include also the term "reagent" or "reagent grade." USP may supply reagents if they otherwise may not be generally commercially available.

6.80. Equipment

Unless otherwise specified, a specification for a definite size or type of container or apparatus in a procedure is given solely as a recommendation. Other dimensions or types may be used if they are suitable for the intended use.

6.80.10. Apparatus for Measurement

Where volumetric flasks or other exact measuring, weighing, or sorting devices are specified, this or other equipment of at least equivalent accuracy shall be employed.

6.80.10.1. Pipet/Pipette

Where a pipet/pipette is specified, a suitable buret may be substituted. Where a "to contain" pipet/pipette is specified, a suitable volumetric flask may be substituted.

6.80.10.2. Light Protection

Where low-actinic or light-resistant containers are specified, either containers specially treated to protect contents from light or clear containers that have been rendered opaque by application of a suitable coating or wrapping may be used.

6.80.20. Instrumental Apparatus

An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics shall be qualified as appropriate. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

6.80.20.1. Chromatographic Tubes and Columns

The term "diameter" refers to internal diameter (ID).

6.80.20.2. Tubing

The term "diameter" refers to outside diameter (OD).

6.80.20.3. Steam Bath

Where use of a steam bath is directed, use actively flowing steam or another regulated heat source controlled at an equivalent temperature.

6.80.20.4. Water Bath

A water bath requires vigorously boiling water unless otherwise specified.

6.80.30. Temperature Reading Devices

Temperature reading devices suitable for pharmacopeial tests conform to specifications that are traceable to a National Institute of Standards and Technology (NIST) standard or equivalent. Temperature reading devices may be of the liquid-in-glass type or an analog or digital temperature indicator type, such as a resistance temperature device, thermistor, or thermocouple. Standardization of thermometers is performed on an established testing frequency with a temperature standard traceable to NIST. For example, refer to the current issue of American Society of Testing and Materials (ASTM) standards E1 for liquid-in-glass thermometers.

7. TEST RESULTS

7.10. Interpretation of Requirements

Analytical results observed in the laboratory (or calculated from experimental measurements) are compared with stated acceptance criteria to determine whether the article conforms to compendial requirements.

The reportable value, which often is a summary value for several individual determinations, is compared with the acceptance criteria. The reportable value is the end result of a completed measurement procedure, as documented.

Where acceptance criteria are expressed numerically herein through specification of an upper and/or lower limit, permitted values include the specified values themselves, but no values outside the limit(s). Acceptance criteria are considered significant to the last digit shown.

7.10.5. Nominal Concentrations in Equations

Where a "nominal concentration" is specified, calculate the concentration based on the label claim. In assay procedures, water correction is typically stated in the Definition and on the label of the USP Reference Standard. For other procedures, correction for assayed content, potency, or both is made prior to using the concentration in the equation provided in the monograph.

7.10.10. Equivalence Statements in Titrimetric Procedures

The directions for titrimetric procedures conclude with a statement of the weight of the analyte that is equivalent to each mL of the standardized titrant. In such an equivalence statement, the number of significant figures in the concentration of the titrant should be understood to correspond to the number of significant figures in the weight of the analyte. Corrections to calculations based on the blank determination are to be made for all titrimetric assays where appropriate (see *Titrimetry* ⟨541⟩).

7.20. Rounding Rules

The observed or calculated values shall be rounded off to the number of decimal places that is in agreement with the limit expression. Numbers should not be rounded until the final calculations for the reportable value have been completed. Intermediate calculations (e.g., slope for linearity) may be rounded for reporting purposes, but the original (not rounded) value should be used for any additional required calculations. Acceptance criteria are fixed numbers and are not rounded.

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is equal to or greater than 5, it is eliminated and the preceding digit is increased by 1.

8. TERMS AND DEFINITIONS

8.10. Abbreviations

- RS refers to a USP Reference Standard.
- CS refers to a Colorimetric Solution.
- TS refers to a Test Solution.
- VS refers to a Volumetric Solution that is standardized in accordance with directions given in the individual monograph or in the *Reagents, Indicators, and Solutions* section of *USP–NF*.

8.20. About

"About" indicates a quantity within 10%.

If the measurement is stated to be "accurately measured" or "accurately weighed," follow the statements in *Volumetric Apparatus* ⟨31⟩ and *Balances* ⟨41⟩, respectively.

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Illustration of Rounding Numerical Values for Comparison with Requirements			
Compendial Requirement	**Unrounded Value**	**Rounded Result**	**Conforms**
Assay limit ≥98.0%	97.96%	98.0%	Yes
	97.92%	97.9%	No
	97.95%	98.0%	Yes
Assay limit ≤101.5%	101.55%	101.6%	No
	101.46%	101.5%	Yes
	101.45%	101.5%	Yes
Limit test ≤0.02%	0.025%	0.03%	No
	0.015%	0.02%	Yes
	0.027%	0.03%	No
Limit test ≤3 ppm	3.5 ppm	4 ppm	No
	3.4 ppm	3 ppm	Yes
	2.5 ppm	3 ppm	Yes

8.30. Alcohol Content

Percentages of alcohol, such as those under the heading *Alcohol Content*, refer to percentage by volume of C_2H_5OH at 15.56°. Where a formula, test, or assay calls for alcohol, ethyl alcohol, or ethanol, the *USP* monograph article Alcohol shall be used. Where reference is made to "C_2H_5OH," absolute (100%) ethanol is intended. Where a procedure calls for dehydrated alcohol, alcohol absolute, or anhydrous alcohol, the *USP* monograph article Dehydrated Alcohol shall be used.

8.40. Atomic Weights

Atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those established by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

8.50. Blank Determinations

Where it is directed that "any necessary correction" be made by a blank determination, the determination shall be conducted using the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted.

8.60. Concomitantly

"Concomitantly" denotes that the determinations or measurements are to be performed in immediate succession.

8.70. Desiccator

The instruction "in a desiccator" indicates use of a tightly closed container of suitable size and design that maintains an atmosphere of low moisture content by means of a suitable desiccant such as anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, or silica gel. See also section *8.220 Vacuum Desiccator*.

8.80. Logarithms

Logarithms are to the base 10.

8.90. Microbial Strain

A microbial strain cited and identified by its American Type Culture Collection (ATCC) catalog number shall be used directly or, if subcultured, shall be used not more than five passages removed from the original strain.

8.100. Negligible

"Negligible" indicates a quantity not exceeding 0.50 mg.

8.110. NLT/NMT

"NLT" means "not less than." "NMT" means "not more than."

8.120. Odor

"Odorless," "practically odorless," "a faint characteristic odor," and variations thereof indicate evaluation of a suitable quantity of freshly opened material after exposure to the air for 15 minutes. An odor designation is descriptive only and should not be regarded as a standard of purity for a particular lot of an article.

8.130. Percent

"Percent" used without qualification means:

- For mixtures of solids and semisolids, percent weight in weight;
- For solutions or suspensions of solids in liquids, percent weight in volume;
- For solutions of liquids in liquids, percent volume in volume;
- For solutions of gases in liquids, percent weight in volume.

For example, a 1 percent solution is prepared by dissolving 1 g of a solid or semisolid, or 1 mL of a liquid, in sufficient solvent to make 100 mL of the solution.

8.140. Percentage Concentrations

Percentage concentrations are expressed as follows:

- *Percent Weight in Weight* (w/w) is defined as the number of g of a solute in 100 g of solution.
- *Percent Weight in Volume* (w/v) is defined as the number of g of a solute in 100 mL of solution.
- *Percent Volume in Volume* (v/v) is defined as the number of mL of a solute in 100 mL of solution.

8.150. Pressure

Pressure is determined by use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

8.160. Reaction Time

Reaction time is 5 minutes unless otherwise specified.

8.170. Specific Gravity

Specific gravity is the weight of a substance in air at 25° divided by the weight of an equal volume of water at the same temperature.

8.180. Temperatures

Temperatures are expressed in centigrade (Celsius) degrees, and all measurements are made at 25° unless otherwise indicated. Where moderate heat is specified, any temperature not higher than 45° (113° F) is indicated.

8.190. Time

Unless otherwise specified, rounding rules, as described in section *7.20 Rounding Rules*, apply to any time specified.

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8.200. Transfer

"Transfer" indicates a quantitative manipulation.

8.210. Vacuum

"Vacuum" denotes exposure to a pressure of less than 20 mm of mercury (2.67 kPas), unless otherwise indicated.

8.220. Vacuum Desiccator

"Vacuum desiccator" indicates a desiccator that maintains a low-moisture atmosphere at a reduced pressure of not more than 20 mm of mercury (2.67 kPas) or at the pressure designated in the individual monograph.

8.230. Water

8.230.10. Water as an Ingredient in an Official Product

As an ingredient in an official product, water meets the requirements of the appropriate water monograph in *USP* or *NF*.

8.230.20. Water in the Manufacture of Official Substances

When used in the manufacture of official substances, water shall meet the requirements for drinking water as set forth in the U.S. Environmental Protection Agency National Primary Drinking Water Regulations or in the drinking water regulations of the European Union or of Japan, or in the World Health Organization's Guidelines for Drinking Water Quality. Additional specifications may be required in monographs.

8.230.30. Water in a Compendial Procedure

When water is called for in a compendial procedure, the *USP* monograph article Purified Water shall be used unless otherwise specified. Definitions for other types of water are provided in *Reagents, Indicators, and Solutions* and in *Water for Pharmaceutical Purposes* ⟨1231⟩.

8.240. Weights and Measures

In general, weights and measures are expressed in the International System of Units (SI) as established and revised by the *Conférence générale des poids et mesures*. For compendial purposes, the term "weight" is considered to be synonymous with "mass."

Molality is designated by the symbol *m* preceded by a number that represents the number of moles of the designated solute contained in 1 kilogram of the designated solvent.

Molarity is designated by the symbol M preceded by a number that represents the number of moles of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

Normality is designated by the symbol N preceded by a number that represents the number of equivalents of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

The symbol for degrees (°) without a qualifying unit of measure represents degrees Celsius.

Chart of Symbols and Prefixes commonly employed for SI metric units and other units:

	Units	Symbol	Notes
Length			
	meter	m	
	centimeter	cm	
	millimeter	mm	
	micrometer	µm	Previously referred to as a micron
	nanometer	nm	Previously the symbol mµ (for millimicron) was used
	Ångström	Å	Equal to 0.1 nm
Mass			
	kilogram	kg	
	gram	g	
	milligram	mg	
	microgram	µg	The symbol µg is used in the *USP* and *NF* to represent micrograms, but micrograms may be represented as "mcg" for labeling and prescribing purposes. The term "gamma," symbolized by γ, frequently is used to represent micrograms in biochemical literature.
	nanogram	ng	
	picogram	pg	
	dalton	Da	Also referred to as the unified atomic mass unit and is equal to 1/12 times the mass of the free carbon 12 atom.
	kilodalton	kDa	
Time			
	second	s	
	minute	min	
	hour	h	
Volume			
	liter	L	1 L is equal to 1000 cm^3 (cubic centimeters)
	deciliter	dL	
	milliliter	mL	1 mL is equal to 1 cm^3, sometimes referred to as cc
	microliter	µL	
Temperature			
	Celsius	°C	
Amount of Substance			
	mole	mol	Historically referred to as gram-molecular weight or gram-atomic weight
	millimole	mmol	
	micromole	µmol	
	femtomole	fmol	
	equivalent	Eq	Also referred to as gram-equivalent weight. It is used in the calculation of substance concentration in units of normality. This unit is no longer preferred for use in analytical chemistry or metrology.
	milli equivalent	mEq	
	osmole	Osmol	Osmotic pressure of a solution, related to substance concentration.
	milliosmole	mOsmol	
Pressure			
	pascal	Pa	
	kilopascal	kPa	

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	Units	Symbol	Notes
	pounds per square inch	psi	
	millimeter of mercury	mmHg	Equal to 133.322 Pa
Electrical units			
	ampere	A	
	volt	V	
	millivolt	mV	
	hertz	Hz	Unit of frequency
	kilohertz	kHz	
	megahertz	MHz	
	electron volt	eV	
	kilo-electron volt	keV	
	mega-electron volt	MeV	
Radiation			
	becquerel	Bq	SI unit of activity for radionuclides
	kilobecquerel	kBq	
	megabecquerel	MBq	
	gigabecquerel	GBq	
	curie	Ci	Non-SI unit of activity for radionuclides
	millicurie	mCi	
	microcurie	μCi	
	nanocurie	nCi	
Other			
	acceleration due to gravity	g	Used to express rate of centrifugation
	revolutions per minute	rpm	Used to express rate of centrifugation

Selected SI Prefixes

Name	Symbol	Factor
giga	G	10^9
mega	M	10^6
kilo	k	10^3
deci	d	10^{-1}
centi	c	10^{-2}
milli	m	10^{-3}
micro	μ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}

9. PRESCRIBING AND DISPENSING

9.10. Use of Metric Units

Prescriptions for compendial articles shall be written to state the quantity and/or strength desired in metric units unless otherwise indicated in the individual monograph [see also section *5.50.10 Units of Potency (Biological)* above]. If an amount is prescribed by any other system of measurement, only an amount that is the metric equivalent of the prescribed amount shall be dispensed. Abbreviations for the terms "Units" or "International Units" shall not be used for labeling or prescribing purposes. Apothecary unit designations on labels and labeling shall not be used.

9.20. Changes in Volume

In the dispensing of prescription medications, slight changes in volume owing to variations in room temperatures may be disregarded.

10. PRESERVATION, PACKAGING, STORAGE, AND LABELING

10.10. Packaging and Storage

All articles in *USP* or *NF* are subject to the packaging and storage requirements specified in *Packaging and Storage Requirements* ⟨659⟩, unless different requirements are provided in an individual monograph.

10.20. Labeling

All articles in *USP* or *NF* are subject to the labeling requirements specified in *Labeling* ⟨7⟩, unless different requirements are provided in an individual monograph.

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General Information Chapters

General Information

The chapters in this section are information, and aside from excerpts given herein from Federal Acts and regulations that may be applicable, they contain no standards, tests, assays, nor other mandatory specifications, with respect to any Pharmacopeial articles. The excerpts from pertinent Federal Acts and regulations included in this section are placed here inasmuch as they are not of Pharmacopeial authorship. Revisions of the federal requirements that affect these excerpts will be included in *USP Supplements* as promptly as practical. The official requirements for Pharmacopeial articles are set forth in the *General Notices,* the individual monographs, and the *General Tests and Assays* chapters of this *Pharmacopeia.*

⟨1004⟩ MUCOSAL DRUG PRODUCTS—PERFORMANCE TESTS

INTRODUCTION

Mucosal drug products deliver drug substances to the body via the mucosal route. For the purposes of this chapter, the mucosal route of drug administration is divided into seven membrane surfaces characterized as otic, ophthalmic, nasal, oropharyngeal, urethral, vaginal, and rectal. Mucosal drug products include a wide variety of dosage forms such as solutions, suspensions, emulsions, creams, ointments, gels, inserts, strips, aerosols, sprays, films, medicated chewing gums, lozenges, tablets, and suppositories. Some of these dosage forms are also administered by other routes. For example, creams can be administered by the mucosal route (vaginal) and also by the topical route. Two categories of tests—product quality and product performance—are performed on these products. These tests provide assurances of batch-to-batch quality, reproducibility, reliability, and performance of a drug product. Product quality tests are performed to assess attributes such as assay, identification, and content uniformity and are part of the compendial monograph (see *Mucosal Drug Products—Product Quality Tests* ⟨4⟩). Product performance tests are conducted to assess the drug release from the dosage form. For certain mucosal drug products, determination of aerodynamic particle size or globule size may serve as a product performance test.

Where a compendial performance test exists for a dosage form administered by a nonmucosal route, such as a dissolution test for an oral tablet, that test may have application for the dosage form administered by a mucosal route (e.g., buccal tablets or sublingual tablets).

PERFORMANCE TESTS FOR MUCOSAL DRUG PRODUCTS

The performance tests for the various mucosal drug products can be broadly divided into two categories: 1) test procedures that use or can adopt methodology in existing general chapters, and 2) tests that need additional developmental work before they can be recommended.

The Dissolution Procedure: Development and Validation ⟨1092⟩ should be a reference when developing a drug release test (e.g., selecting the drug release medium, apparatus/procedure, or analytical method). For several mucosal drug products, drug release procedures described in *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, and *Semisolid Drug Products—Performance Tests* ⟨1724⟩ may be applicable.

In some instances, mini-basket or mini-paddle apparatuses may be suitable. These apparatuses resemble *Dissolution* ⟨711⟩, *Apparatus 1 (Basket Apparatus)* and *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)*, with dimensions scaled down to accommodate medium volumes of <500 mL (*1*,*2*). Several designs are commercially available. However, as of now, these apparatuses are not standardized.

Because of the varied and specific environments characterizing the mucosal route of administration, researchers may be inclined to use "physiological medium" for the drug release of the specific dosage form. It may not be essential to use such a medium for the performance test of the product, and in many instances a simple buffer may suffice. A reference (*3*) for the composition of such media is provided for additional information.

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Aerosols and Nasal Sprays

Performance tests for nasal and lingual aerosols and nasal sprays are largely concerned with droplet or particle size distribution and aerodynamic size distribution. The procedures in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ can be applied to products administered by mucosal routes. When the drug substance is present as a solid with a modified-release mechanism in the administered dose, attempts should be made to determine the dissolution of the particles (*4*).

Creams, Gels, and Ointments

Drug release tests for creams, gels, and ointments can be performed using a procedure described in ⟨1724⟩.

Emulsions

Performance tests for emulsions include globule size determination and dissolution/drug release testing. Globule size can be determined using a procedure described in *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩. The drug release test can be performed using *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)* or a vertical diffusion cell as described in *Semisolid Drug Products—Performance Tests* ⟨1724⟩, *Drug Release Rate Determination Using Vertical Diffusion Cell Apparatus*.

Films

Drug Release ⟨724⟩, *Apparatus 5 (Paddle over Disk)* can be used to determine the drug release from film dosage forms. A mini-basket can be used for drug release testing of films.

Gums

For gum products, the performance test includes drug release from the formulation. A device is described in the *European Pharmacopoeia* (*5*). The release of drug from the formulation requires masticatory activity that renews the surface exposed to the medium. The rate of release will be a function in part of the frequency of chewing that is simulated by the test apparatus. Gums can require conditioning at the temperature of the mouth to deform plastically under the action of the oscillating platens of the test apparatus.

Important parameters for the apparatus include: dissolution medium volume, distance between upper and lower chewing surfaces, recommended rotation angle, temperature, and chewing frequency. The dissolution medium chosen, the test time(s), and the volume sampled are also important considerations.

Inserts

Drug release testing for inserts can be performed using *Dissolution* ⟨711⟩, *Apparatus 1 (Basket Apparatus)* or *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)*.

Lozenges

Drug release testing of lozenges can be performed using either *Dissolution* ⟨711⟩, *Apparatus 1 (Basket Apparatus)*; *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)* at high agitation (175 rpm); or *Dissolution* ⟨711⟩, *Apparatus 3 (Reciprocating Cylinder)*.

Suppositories

There are two types of suppositories: 1) hydrophilic (water soluble), and 2) lipophilic (oil soluble or melting). Drug release (dissolution) for water-soluble suppositories can be performed using *Dissolution* ⟨711⟩, *Apparatus 1 (Basket Apparatus)*; *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)*; or *Dissolution* ⟨711⟩, *Apparatus 4 (Flow-Through Cell)*. Drug release testing for lipophilic suppositories may need modification of the dissolution procedure to avoid analytical interference from the oil globules. Several alternative methods have been proposed (*6–8*). The flow-through cell apparatus using the cell for suppositories may be useful. The selection of the method will be dependent on the nature of the formulation. *Figure 1* shows the schematic view of a flow-through cell [*Dissolution* ⟨711⟩, *Apparatus 4 (Flow-Through Cell)*] specifically intended for dissolution of suppositories. The lower part (1) is made up of two adjacent chambers connected to an overflow device. The dissolution medium passes through chamber A and is subjected to an upward flow. The flow in chamber B is directed downward to a small-size bore exit that leads upward to a filter assembly. The middle part (2) of the cell has a cavity designed to collect lipophilic excipients that float on the dissolution medium. A metal grid serves as a rough filter. The upper part (3) holds a filter unit for paper, glass fiber, or cellulose filters.

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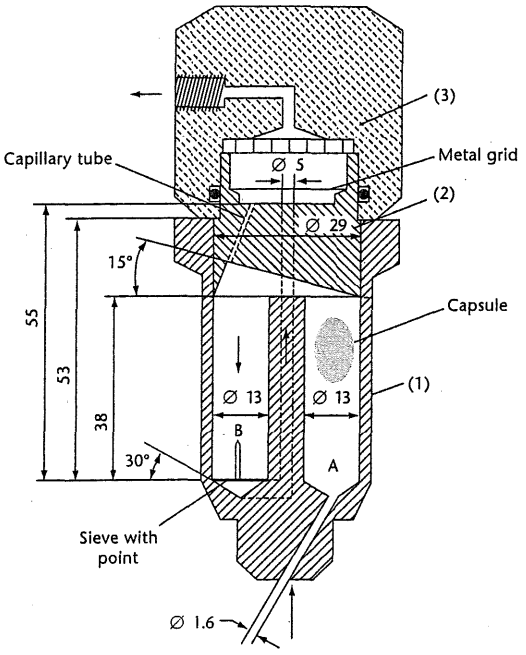

Figure 1. Flow-through cell designed for suppositories (dimensions in mm).

Suspensions

The dissolution test for suspensions can be performed using *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)*. A small-volume, mini-paddle apparatus may be used.

Sublingual Tablets and Buccal Tablets

Drug release for these dosage forms can be performed using *Dissolution* ⟨711⟩, *Apparatus 1 (Basket Apparatus)* or *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)*. Mini-baskets or the mini-paddles can also be used for drug release testing of buccal and sublingual tablets.

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⟨1005⟩ ACOUSTIC EMISSION

INTRODUCTION

Ultrasound techniques can be categorized into two distinct types: acoustic emission (passive mode) and ultrasound spectroscopy (active mode). Both of these techniques have many applications.

The technique of acoustic emission is based on the detection and analysis of sound produced by a process or system. This is essentially equivalent to listening to the process or system, although these sounds are often well above the frequencies that can be detected by the human ear. Generally, frequencies up to about 15 kHz are audible.

In the case of ultrasound spectroscopy, the instrument is designed to generate ultrasound waves across a defined frequency range. These waves travel through the sample and are measured using a receiver. An analogy can be drawn with UV-visible or IR spectroscopy in that the detected ultrasound spectrum reflects changes in velocity or sound attenuation due to the interaction with a sample across a range of frequencies. However, as the scope of this chapter is limited to acoustic emission, ultrasound spectroscopy will not be discussed further.

Acoustic emission is well-known in the study of fracture mechanics and therefore is used extensively by material scientists. It is also widely used as a nondestructive testing technique and is applied routinely for the inspection of aircraft wings, pressure vessels, load-bearing structures, and components. Acoustic emission is also used in the engineering industry for the monitoring of machine tool wear.

In terms of pharmaceutical applications, the dependence of the acoustic emission measurement on physical properties such as particle size, mechanical strength, and cohesivity of solid materials allows the technique to be used for the control and endpoint detection of processes such as high shear granulation, fluid bed drying, milling, and micronization.

General Principles

Acoustic emissions can propagate by a number of modes. In solids, compressional and shear or transverse modes are important. Compressional modes have the highest velocity and thus reach the acoustic detector (or acoustic emission transducer) first. However, in most process applications of acoustic emission, there are many sources—each producing short bursts of energy—and, consequently, the different modes cannot easily be resolved. The detected signal, for example on the wall of a vessel, is a complex mixture of many overlapping waveforms resulting from many sources and many propagation modes.

At interfaces, depending on the relative acoustic impedance of the two materials, much of the energy is reflected back towards the source. In a fluidized bed, for example, acoustic emissions will only be detected from particles directly impacting the walls of the bed close to the transducer.

A convenient method of studying acoustic emission from processes is to use the "average signal level". A root mean square-to-direct current (RMS-to-DC) converter may be used to convert the amplitude-modulated (AM) carrier into a more slowly varying DC signal. This is referred to as the average signal level (ASL). The ASL can then be digitally sampled (typically at a sampling frequency of about 50 Hz) and stored electronically for further signal processing.

The simplest way of studying the acoustic data is to examine changes in the ASL. However, other information can be derived from examining the power spectrum of the ASL. The power spectrum is calculated by taking the complex square of the amplitude spectrum and can be obtained by performing a Fast Fourier Transform (FFT) on the digitized raw data record. Power spectra may be averaged to produce a reliable estimate of power spectral density or to give a "fingerprint" of a particular process regime. Interpretation of the power spectrum is complicated by the fact that the acoustic signal originating in the system is distorted by several factors including transmission, reflection, and signal transfer characteristics.

The shape of the power spectrum of the ASL record is a function of the process dynamics. Periodic processes (e.g., mechanical stirring or periodic bubbling of a fluidized bed) show high power at certain discrete frequencies. Random processes show either flicker type properties, where power is inversely proportional to frequency, or white noise type properties in which power is independent of frequency. The amplitude of the power spectrum is also affected by the energy of the acoustic emissions produced by the process. For example, if hard material is being processed, the acoustic emission produced by particle impact will be greater than that produced by soft material.

INSTRUMENTATION

Generally, piezoelectric sensors are used to detect and quantify the acoustic signals produced by a process. Piezoelectric transducers are constructed from piezoelectric crystalline solids connected to transducer control circuitry by electrical leads. When configured as a detector, an acoustic wave that impinges on the piezoelectric element is transformed into an electrical signal in the transducer control circuitry. When configured as an acoustic generator, an electrical signal applied to the piezoelectric element by the control circuitry creates an acoustic wave that can propagate into the medium to which the transducer is attached. Typically, this means that acoustic emission detectors can also be operated as acoustic wave generators and this feature is used to ensure good sensor performance as described later (see *Qualification and Verification of Acoustic Emission Instruments*).

In general acoustic emission applications, sensors with different resonance frequencies are often used (e.g., 70 and 190 kHz, although higher frequencies may be more appropriate at smaller scales of operation), incorporating various band-passes. As sound (ultrasound) of the appropriate frequency range reaches these sensors, an electrical signal is generated, the amplitude of which is directly proportional to the energy (amplitude) of the incident sound waves.

These signals are processed through the following:

1. a pre-amplifier (which incorporates signal filtering),

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2. an RMS-to-DC converter,
3. a variable gain amplifier, and
4. a PC-based data acquisition board.

The controlling software is also incorporated into the PC.

Acoustic emission equipment generally allows several sensors to be used simultaneously by incorporating multiple electronic channels into a single instrument.

Signal Processing

The signal from a resonant transducer resembles an AM radio signal. At the resonance frequency of the transducer, the signal consists of a carrier wave that is modulated in amplitude by the process. An RMS-to-DC converter is used to demodulate the signal. The output of this device is the modulation signal or envelope.

The envelope is digitally resampled at a frequency appropriate for the process. For example, 50 Hz is a typical digital sampling rate for a fluid bed drier or high shear granulator.

FACTORS AFFECTING MEASUREMENT

The following factors can affect the acoustic data obtained and should be considered when installing an acoustic emission system.

1. *Failure or Physical Damage*—As with any other type of sensor, acoustic emission sensors can fail with time or as a result of physical damage. It is important to check the sensor function as part of routine maintenance of the instrument. If multiple sensors are installed on the same vessel, an active signal can be generated from one sensor and this can be used to check the detection on another sensor. This exercise would ensure that the sensors are detecting the acoustic signals generated by the process. A statistically valid "minimum acceptable acoustic signal" for the sensor(s) should also be determined and monitored at the start, middle, and end of a process to ensure the performance of the sensor(s) during a process run. This may be established from the routine maintenance signal experiments or on the basis of historical data for the sensors.
2. *Issues of Sensor Interfacing*—Sensors are typically installed on the outer wall of the process vessel. Several types of adhesives (temporary or permanent) can be used to attach the sensor to the vessel wall. Through repeated cleaning and vessel movement, it is possible for the bonding between the sensor and vessel to be compromised. Checking the integrity of the installation should be part of routine maintenance. Similar to item 1 above, an active signal can be used to ensure proper bonding between sensor and vessel and helps to confirm the matching of acoustic impedance.
3. *Influence of Mechanical Noise*—The use of high frequencies significantly reduces the contribution of mechanical noise to the acoustic signal detected, especially at smaller scales of operation, although it does not eliminate it completely. Testing the effect of various motor settings, for example, can determine if the acoustic signal detected is a function of mechanical noise. If the effect is significant, using higher frequencies may be necessary. Awareness of the contribution of the mechanical noise, no matter how small, is important to consider as the motors age or are replaced.
4. *Influence of Vessel Wall Characteristics*—Because the sensors are often placed on the outer vessel wall, wall thickness can affect the quality of the signal detected. If the vessel is jacketed, the amplitude of the acoustic signal may be reduced. Adding more sensors on the vessel can improve signal quality. Alternatively, an increase in signal may be obtained by positioning sensor(s) at a location where contact exists between the inner and outer walls, essentially providing a waveguide between the sensor and sound source. Waveguides may also be incorporated into the design of manufacturing equipment to enable utilization of acoustic emission monitoring. Appropriate validation is required to ensure that this does not adversely affect the performance of equipment.
5. *Effect of Material Properties*—During operation, the acoustic signal collected is a summation of various events occurring within the process. For example, the acoustic signal generated as particles hit the wall in a granulator is a function of the material properties of the granules (i.e., density, size, porosity). Therefore, significant changes to any of these parameters can affect the acoustic signal and the quality of the ensuing prediction.
6. *Influence of Process-Related Factors*—Similar to item 5 above, the process-related properties (i.e., force of impact, frequency of impact, amount of material) can also affect the acoustic signal and the quality of the ensuing prediction.
7. *Impact of Environmental Conditions*—Finally, the influence of environmental factors (i.e., temperature, humidity) must also be considered.

The acoustic emission data collected is vessel/equipment specific. It is not advisable to apply a model generated on one piece of equipment to another because the acoustic information can differ as a result of the issues discussed in items 3, 4, and 5 above.

Qualification and Verification of Acoustic Emission Instruments

A system suitability approach should be taken around instrument performance, establishing optimum measurement configuration, then comparing the instrument performance to the values obtained during routine use to those obtained during installation qualification (IQ).

This approach effectively answers the issues related to sampling because, unlike other on-line analytical systems, the transducers can be optimally positioned and attached to receive the maximum signal without vessel modification.

Sample rates need to comply with the Nyquist sampling theorem, which states that a signal must be sampled at a rate that is twice the highest frequency component in the signal. A low-pass filter should be used to remove the frequency components greater than half the sampling frequency (Nyquist frequency). Failure to comply with this criterion will result in aliasing.

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Owing to the nature of the piezoelectric transducers and because resonance frequencies are natural properties of the crystals, it is not necessary to test the variation (reproducibility) or drift in the frequency domain. If other types of transducers are used, this may be necessary. Any gross change in the frequency domain will be recorded as a drop in the power intensity at the resonance frequency, and therefore is covered by the power intensity tests.

The two main areas for instrument performance verification are power intensity and timings. Any change in the signal intensity will affect the raw signal and the ASL and, therefore, will also affect the power spectrum. Changes in power intensity can occur as a result of changes in the process (e.g., variation in hardness or moisture in the particles impacting the vessel wall) or changes in the acoustic conduit from the process to transducer.

Reproducibility of the acoustic conduit should be tested using a second transducer to input a pulse or "ping" at the resonance frequency of the receiving sensor. This reproducibility value represents the noise of the signal and can be used in calculations of limit of detection (LOD) and limit of quantitation (LOQ), where LOD is defined as three times the noise of the signal and LOQ is ten times the noise of the signal. The noise on the background signal level (in acoustic emission this background signal is mainly due to amplifier noise) should be calculated from twenty sequential ASL values acquired at the sampling frequency used for normal operation. This test should be repeated in reverse in order to establish that statistically similar intensity values can be obtained on both channels.

Short term reproducibility allows the calculation of noise. However, it does not give a measure of integrity of acoustic conduit over time or, more specifically, of changes caused by the process (e.g., variations in adhesive properties with process changes such as heating/cooling). The noise test should be repeated while executing the normal processing parameters (using an empty vessel) and the drift in the ASL should be calculated. Care should be taken to make sure that signal drift (due to normal variation in processing parameters) does not impact chemometric models used for endpoint determination. For trend plots, it should be shown that drift is not statistically significant; otherwise, drift correction will need to be applied. Values for noise, drift, and absolute ASL should be recorded and logged, and the tests re-executed if changes are made to the processing equipment or to the acoustic emission system. If no changes are made, then the tests should be re-executed every month. In this way the quality of the acoustic conduit can be shown to be intact and any changes to the signal intensity isolated and attributed to the process itself.

During routine use, it is recommended that the noise test be executed (as above) before each process run, and that power intensity and noise be calculated. These values should be logged and compared to those generated both during previous use and during installation. Impact of the deviation from previous values will be a function of the prediction model and should be addressed by method validation.

The noise data (from above) can also be used to calculate the time of flight of the pulse. If the pulse activation and signal reception are synchronized, the time taken for the pulse to transmit across the vessel can be measured. This is a good indication of the measurement electronics as well as the overall condition of the acoustic conduit. However, this test should be regarded as a measure of the "system" condition and needs to be executed only if changes have been made to the process equipment or the acoustic emission system, or every 6 months. Correlation of the measured timings with the historical ones should be statistically valid. If not, it is an indicator that the acoustic emission system may need requalification by the instrument manufacturer or supplier, or that there are changes in the acoustic conduit.

All of these tests require the use of an acoustic pulse generated electrically. Failure in any of the above tests could be attributed to the signal generation itself. It is recommended that the electrical pulse generation system be requalified and certified against National Institute of Standards and Technology (NIST) traceable standards every 12 months.

DATA ANALYSIS

Acoustic emission from granulators and fluid bed driers is known as continuous acoustic emission. Continuous acoustic emission is aphasic (i.e., there are no starts or stops to the signal). This means that it is unnecessary to use signal processing techniques that preserve phase. Power spectral analysis is a useful technique in processing acoustic emission signals. The information in the power spectra, unlike the raw acoustic emission signals, is coherent in the short term, allowing signal averaging to be performed. This provides a better estimate of power spectral density than that provided by a single power spectrum.

To detect endpoints in batch processes (e.g., granulation or drying endpoint), a qualitative multivariate model is appropriate (e.g., PCA or SIMCA). The following sequence of operations is performed:

1. *Training/Calibration*—Acoustic emission spectra that are representative of the endpoint condition are obtained.
2. *Modeling*—A multivariate model describing the distribution of acoustic emission signals at the endpoint condition is created.
3. *Prediction*—Acoustic emission spectra are compared against the model. The fit to the model (usually expressed in terms of a number of standard deviations) is monitored. As the system approaches the endpoint, the fit improves and completion of the process is established once the model fits predefined criteria. The prediction model is generated from acoustic emission spectra obtained from the process operating under normal conditions. Upsets (e.g., unwanted agglomeration in coaters) are detected by observing statistically valid deviations from the model.
   Adaptive modeling has also been proposed for upset detection. This involves generating multivariate models continuously as the acoustic emission signals are acquired. Unusual deviation of the acoustic emission signal indicates the occurrence of a process upset. The advantage of adaptive modeling is that it is not necessary to perform a separate calibration step.

GLOSSARY

Acoustic Emission Transducer: A solid state device usually incorporating a piezoelectric element to convert the acoustic emission wave to an electrical signal.

Acoustic Impedance: Acoustic impedance (Z) is defined as $Z = \rho v$ (where ρ is density and v is the sound velocity). It is an important quantity and gives the proportion of sound energy transmitted from one medium to another and the amount of energy reflected at the interface.

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Adaptive Modeling: A method that predicts the state of a process without the use of a previously generated model (i.e., there is no prior training or calibration step).

Aliasing: Spurious low frequency components, appearing in the signal, that are really frequencies above the Nyquist frequency.

Amplitude: The magnitude or strength of a varying waveform.

Average Signal Level (ASL): A measure of the average power in an acoustic emission signal.

Band-Pass: The range of frequencies within which a component operates.

Compressional Mode: A longitudinal mode of acoustic transmission encountered in solids, liquids, and gases.

Continuous Acoustic Emission: Acoustic emission signals that cannot be separated in time and are typical of pharmaceutical processes such as granulation and fluid bed drying.

Flicker Type Properties: A type of signal associated with many natural processes. The characteristics of flicker noise are that the power of the noise is directly proportional to the signal and has approximately a 1/f (f = frequency) spectral density distribution.

Gain: The amplification factor for a component usually expressed in terms of decibels (dB).

Gain in dB = 20 $\log_{10}$ ($Voltage_{out}$ $Voltage_{in}$).

Nyquist Frequency: The Nyquist frequency is defined as half the digital sampling rate and is the highest frequency that can be reproduced faithfully.

Piezoelectric: A material which generates an electric field when compressed. Piezoelectric materials are used in the construction of acoustic emission sensors. A common material is PZT (lead zirconium titanate).

Power Spectrum: A power spectrum of a signal is a representation of the signal power as a function of frequency. A power spectrum is calculated from the time domain signal by means of the Fast Fourier Transform (FFT) algorithm. It is useful to study acoustic emission signals in the frequency or spectral domain, as the spectrum is often characteristic of the mechanism. Improvements in signal-to-noise ratio can be obtained by averaging a number of power spectra, as they are coherent.

Power Spectral Density: The measure of acoustic emission power in each resolution element of the power spectrum.

Resonance Frequency: The frequency at which an acoustic emission sensor is most sensitive. Resonant acoustic emission sensors have a clearly defined resonance frequency, but are usually sensitive to other frequencies.

RMS-to-DC Converter: An electronic device that converts an alternating signal to a voltage level proportional to the average power in the signal.

Shear Mode: A transverse mode of acoustic transmission, encountered only in solids.

Signal Filtering: Filtering a signal means attenuating frequencies outside a prescribed range. In acoustic emission work, band-pass filtering is used to improve the signal-to-noise ratio by attenuating noise outside the bandwidth of the sensor. Low-pass filtering is used to remove frequencies higher than the Nyquist frequency in order to prevent aliasing.

Transverse Mode: A mode of wave propagation where the displacement of the material is perpendicular to the direction of propagation. These modes are only encountered in solid materials.

White Noise: The characteristic of white noise is a power spectrum of uniform spectral density and is associated with purely random processes.

⟨1010⟩ ANALYTICAL DATA—INTERPRETATION AND TREATMENT

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▲1. INTRODUCTION

This chapter provides information regarding acceptable practices for the use of analytical procedures to make decisions about pharmaceutical processes and products. Basic statistical approaches for decision making are described, and the comparison of analytical procedures is discussed in some detail.

[NOTE—It should not be inferred that the analysis tools mentioned in this chapter form an exhaustive list. Other, equally valid, statistical methods may be used at the discretion of the manufacturer and other users of this chapter.]

Assurance of the quality of pharmaceuticals is accomplished by combining a number of practices, including rigorous process and formulation design, validation, and development and execution of a robust control strategy. Each of these is dependent on reliable analytical procedures. In the development process, analytical procedures are utilized to ensure that the manufactured products are thoroughly characterized and to optimize the commercial manufacturing process. Final-product testing provides assurance that a product is consistently safe, efficacious, and in compliance with its specifications. Sound statistical approaches can be included in the commercial control strategy to further ensure that quality is preserved throughout the product lifecycle.

While not meant to be a complete account of statistical methodology, this chapter will rely upon some fundamental statistical paradigms. Key among these are *population parameters*, *statistical design and sampling*, and parameter *uncertainty*. Population parameters are the true but unknown values of a scientific characteristic of interest. While unknown, these can be estimated using statistical design and sampling. Statistical design is used to fully represent the population of interest and to manage the uncertainty of a result, while the random acquisition of test samples as well as their introduction into the measurement process helps to mitigate bias. Lastly uncertainty should be acknowledged between the true population parameter and the estimation process. Uncertainty can be expressed as either a probabilistic margin between the true and estimated value of a population parameter (e.g., a 95% confidence interval) or as the certainty that the population parameter is compliant with some expectation or acceptance criterion (predictive probability).

This chapter provides direction for scientifically acceptable administration of pharmaceutical studies using analytical data. Focus is on investigational studies where analytical data are generated from carefully planned and executed experiments, as well as confirmatory studies which are strictly regulated with limited flexibility in design and evaluation. This is in contrast to

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exploratory studies where historical data are utilized to identify trends or effects which are subject to further investigation. The quality of decisions made from either investigational or confirmatory studies is enhanced through adherence to the scientific method, and to the application of sound statistical principles. The steps of the scientific method can be summarized as follows.

Study objective. A pharmaceutical study can be as simple as testing and releasing a batch of commercial material or as complex as a comparison of analytical procedures. The same considerations apply to the simple study as they do to the complex study. Each study is associated with a population parameter which is used to address the study objective. For release the parameter might be the batch mean. For the analytical procedure comparison study, the parameter might be the difference in means produced by the analytical procedures. In each case an appropriate acceptance criterion on the population parameter is used to make a decision from the study.

Study design. The study should be designed with a structure and replication strategy which ensures representative consideration of the study objective, and which manages the risks associated with making an incorrect decision. Representative consideration of the study objective entails inclusion of samples and conditions which span the population being studied. Thus in release of a manufactured lot, samples across the range of manufacture might be included, while in a procedure comparison, each type and level of test sample might be considered. Similar consideration should be given to sample testing, where appropriate factors should be included in the procedure. The design should also acknowledge the study risks. The statistical basis for managing study risk is the reduction of the uncertainty in the estimation of the population parameter.

Study conduct. Once the study has been designed, samples are collected and data are generated using the analytical procedure. Effective use of randomization should be considered to minimize the impact of systematic variability or bias. Care should be taken during data collection to properly control the analytical procedure and to ensure accurate transcription and preservation of information. An adequate number of significant digits or decimal places should be saved and used throughout the calculations. Deviations from the study plan should be captured and assessed for their potential to impact study decisions.

Study analysis and decision. Prior to the final analysis, the data should be explored for data transformation and potential outliers. The analysis of the data should proceed according to the statistical methods considered during the study design. The analysis of the data and the reporting of study results should include proper consideration of uncertainty. Where appropriate, interval estimates should be used to communicate the robustness of the results (viz., the width of the interval) as well as facilitate communication of the study decision. A decision can be made when the objective of the study has been pre-formulated to make such a decision (e.g., as in an investigational or confirmatory study). The study may otherwise have been performed to estimate or describe some characteristic of a population. Caution should be taken in making decisions from post-hoc analyses of the data. This is called "data snooping" and can lead to inappropriate decisions.

This chapter has been written for the laboratory scientist and the statistician alike. The laboratory scientist is primarily skilled in the analytical procedures and the uses made of those procedures and should be aware of the value of statistical design and analysis in their practices. The statistician is primarily skilled in the design of empirical studies and the analysis which will return reliable decisions and should appreciate the science and constraints within the laboratories. While variously knowledgeable in their understanding across specialties, both disciplines should value the essential components that comprise uses of analytical data.

More detailed discussion related to the steps of the scientific method will be given in Section 4, *Study Considerations*, and will be illustrated with an example in Section 5, *Analytical Procedure Comparison*. Prior to this Section 2 will review some *Prerequisite Laboratory Practices and Principles*, and Section 3 will describe and illustrate some *Basic Statistical Principles and Uncertainty*. A series of appendices is provided to illustrate topics related to the generation and use of analytical data. Control charts, equivalence and noninferiority testing, the principle of uncertainty, and Bayesian statistics are briefly discussed. The framework within which the results from a compendial test are interpreted is clearly outlined in *General Notices, 7. Test Results*. Selected references that might be helpful in obtaining additional information on the statistical tools discussed in this chapter are listed in *Appendix 6: References* at the end of the chapter. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about many of the methods cited in this chapter may also be found in most statistical textbooks.

2. PREREQUISITE LABORATORY PRACTICES AND PRINCIPLES

The sound application of statistical principles to analytical data requires the assumption that such data have been collected in a traceable (i.e., documented) and unbiased manner. To ensure this, the following practices are beneficial.

Sound Record Keeping

Laboratory records are maintained with sufficient detail, so that other equally qualified analysts can reconstruct the experimental conditions and review the results obtained. When collecting data, the data should be obtained with more decimal places than the specification or study acceptance criterion requires. Rounding of results from uses of analytical data should occur only after final calculations are completed as per the *General Notices*. Study protocols and data analyses should be adequately documented so that a reviewer can understand the bases of the study design and the pathway to study decisions.

Procedure Validation

Analytical procedures used to release and monitor stability of clinical and commercial materials are appropriately validated as specified in *Validation of Compendial Procedures* ⟨1225⟩ or verified as noted in *Verification of Compendial Procedures* ⟨1226⟩. Further guidance is given in *Statistical Tools for Procedure Validation* ⟨1210⟩ and *Biological Assay Validation* ⟨1033⟩. Analytical procedures published in the *USP–NF* should be validated and meet the Current Good Manufacturing Practices (GMP) regulatory requirement for validation as established in the United States Code of Federal Regulations. When an analytical procedure is used

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in a non-GMP study, it's good practice to ensure that the analytical procedure is adequately fit for use to support the study objective.

Analytical Procedure and Sample Performance Verification

Verifying an acceptable level of performance for an analytical procedure in routine or continuous use is a valuable practice. This may be accomplished by analyzing a control sample at appropriate intervals or locations, or using other means, such as, determining and monitoring variation among the standards, background signal-to-noise ratios, etc. This is commonly called system suitability. Attention to the measured performance attribute, such as charting the results obtained by testing of a control sample, can signal a change in performance that requires adjustment of the analytical system. Examples of control charts used to monitor analytical procedure performance are provided in *Appendix 1: Control Charts*.

Sample performance should also be verified during routine use of an analytical procedure. Variability among replicates as well as other sample specific performance attributes are used to ensure the reliability of sample measurement. A failure to meet a sample performance requirement can result in a retest of the sample after an appropriate investigation, versus a complete repeat of an analytical procedure run.

3. BASIC STATISTICAL PRINCIPLES AND UNCERTAINTY

This section introduces the concept of uncertainty, and couples this with familiar statistical tools which facilitate decisions made from analytical data. At the core of these principles and tools is an understanding of risk; more specifically the risks of making incorrect decisions based on analyses using measurement data. The consequences of these risks can be minor or significant, and should be factored into considerations related to both design of a study, and the interpretation of the results. The understanding of uncertainty is not new to the pharmaceutical industry, or more broadly throughout industries that make decisions from analytical data. The study of measurement and measurement uncertainty falls formally into the field of metrology (see *Appendix 4: The Principle of Uncertainty*). This section will frame the concept of uncertainty and illustrate some well-known statistical tools.

Uncertainty

A study is designed to reduce uncertainty in order to make more reliable decisions.

Uncertainty is associated with variability and communicates the closeness of a result to its true value. A fundamental aspect of uncertainty is probability which is sometimes expressed as confidence. The combination of the variability of the result from a study and confidence provides a powerful means to manage pharmaceutical decisions.

Uncertainty is directly related to risk. Risk may be expressed as a probability, but is more formally translated into cost, where cost is the opportunity loss due to making an incorrect decision times the probability of that loss. Here a loss may be quantifiable outcome such as the value of a lot of manufactured material, or less quantifiable such as the loss of patient benefit from a drug or biological.

Key to the concept of uncertainty is its relationship to the structure of variability. The overall variability of the result is a composition of many individual sources of variability. In a general sense one can manage the overall variability through refinement in one or some of those sources, or through strategic design (e.g., replication and blocking). In either case the effort results in higher certainty and lower risk.

Basic Statistical Principles

All results from studies using analytical data are, at best, estimates of the true value because they contain uncertainty. Basic statistical principles related to estimation and uncertainty will be illustrated for the population mean of a manufactured lot.

STATISTICAL MEASURES

Statistical measures used to estimate the center and dispersion of a population include the mean, standard deviation, and expressions derived there from, such as the percent coefficient of variation (%CV), sometimes referred to as percent relative standard deviation (%RSD). Such statistical measures can be used to calculate confidence intervals for summary parameters of the process generating the data, prediction intervals for capturing a single future measurement with specified confidence, or tolerance intervals capturing a specified proportion of the individual measurements with specified confidence.

STATISTICAL ASSUMPTIONS

Statistical assumptions should be justified with respect to the underlying data generation process and verified using appropriate graphical or statistical tools. If one or more of these assumptions appear to be violated, alternative methods may be required in the evaluation of the data. In particular, most of the statistical measures and tests cited in this chapter rely on the assumptions that the underlying population of measurements is normally distributed and that the measurement results are independent and free from aberrant values or outliers. Assessment of the statistical assumptions and alternatives methods of analysis are discussed in *Appendix 2: Models and Data Considerations*.

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AVERAGING

A single analytical measurement may be useful in decision making if the sample has been prepared using a well-validated documented process, if the sample is representative of the population of interest, if the analytical errors are well known, and the measurement uncertainty associated with the single measurement is suitable to make the appropriate decision. The obtained analytical result may be qualified by including an estimate of the associated measurement uncertainty. For a single measurement this might come from the procedure validation or another source of prior knowledge.

There may be instances when one might consider averaging multiple measurements because the variability associated with the average value better meets the target measurement uncertainty requirement for its use. Thus, the choice of whether to use individual measurements or averages will depend upon the use of the measurement and the risks associated with making decisions from the measurement. For example, when multiple measurements are obtained on the same sample aliquot (e.g., from multiple injections of the sample in an HPLC procedure), it is generally advisable to average the individual values to represent the sample value. This should be supported by some routine suitability check on the variability amongst the individual measures. A decision rule, which defines and describes how a decision will be made, should be explicit to the population parameter of interest. When this is the center or the mean, then the average should be the basis of the rule. When this is variability amongst the individual measurements, then it should be the standard deviation, %CV, or range. Except in special cases (e.g., content uniformity), care should be taken in making decisions from individual measurements.

ESTIMATING THE CENTER AND DISPERSION FROM A SAMPLE

Let $Y_1, Y_2, \ldots, Y_n$ represent a sample of (n) observations from a population of interest. When the appropriate assumptions are met the most commonly used statistic to describe the center of the (n) observations is the sample or arithmetic mean $(\overline{Y})$:

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} = \frac{Y_1 + Y_2 + \ldots + Y_n}{n} \qquad (1)$$

The dispersion can be estimated from the observations in various ways. The most common and useful assessment of the dispersion is the determination of the sample standard deviation. The sample standard deviation is calculated as

$$S = \sqrt{\frac{\sum_{i=1}^{n} (Y_i - \overline{Y})^2}{n-1}} \qquad (2)$$

The sample %CV is calculated as

$$\%\,CV = \frac{S}{\overline{Y}} \times 100\,\% \qquad (3)$$

It should be noted that %CV is an appropriate measure of variability only if the property being measured is an absolute quantity such as mass. It is incorrect to report %CV for estimates reported as a percentage (e.g., percent purity) or which are in transformed units (e.g., pH or other logarithmic units; see Torbeck, 2010).

STATISTICAL INTERVALS

Statistical intervals are used to describe or make decisions concerning population parameters or behavior of individual values. Three useful statistical intervals are prediction intervals, tolerance intervals, and confidence intervals. Prediction and tolerance intervals describe behavior of individual values and are discussed in ⟨1210⟩.

Confidence intervals are the basis for incorporating uncertainty into the estimate of a population parameter. A two-sided interval is composed of a lower bound *LB* and an upper bound *UB*. For a confidence interval on a population parameter θ these bounds are functions of the sample values such that

$$Pr[LB \le \theta \le UB] = 100 \times (1 - \alpha)\% \qquad (4)$$

This leads to the construction of a $100 \times (1 - \alpha)\%$ two-sided confidence interval on a population mean

$$LB = \overline{Y} - t_{1-\alpha/2:n-1}\frac{S}{\sqrt{n}}$$
$$UB = \overline{Y} - t_{1-\alpha/2:n-1}\frac{S}{\sqrt{n}} \qquad (5)$$

where n is the sample size and $t_{1-\alpha/2:n-1}$ is the $1 - \alpha/2^{th}$ quantile of the cumulative Student t distribution having area $1 - \alpha/2$ to the left and $n - 1$ degrees of freedom. One-sided intervals based on the individual bounds can be similarly defined.

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The sampling and calculation process described above will provide a confidence interval that contains the true parametric value $100 \times (1 - \alpha)\%$ of the time. Alternatively one can utilize a Bayesian approach to derive an interval which contains, with probability $100 \times (1 - \alpha)\%$ the true value of the mean (*12*).

4. STUDY CONSIDERATIONS

There are a number of scientific and statistical considerations in conducting a study. These will be discussed in the context of the stages of the scientific method (see *Introduction*).

Study Objective

The study objective is a statement of the goal(s) of the study. Generally, the goals are placed into two categories: (1) estimation, and (2) inference. Estimation is the goal when the investigator wishes to report results that estimate true quantities that underlie the data generating process and are the subject of the study. In statistics these true quantities are called population parameters. Inference includes the additional step of using these estimates to make a decision about the unknown true value of the population parameter.

Numerical estimates can either be single numbers (point estimates), a range of numbers (interval estimates), or distributions (distributional estimates). A point estimate is a single number that "best" represents the unknown true value of a population parameter. The computed average or standard deviation of a data set sampled from the study population are examples of point estimates. "Best" in this context means the estimate is in some sense close to the unknown parameter value, although the difference between the estimate and the parameter will vary from sample to sample.

A point estimate reported alone has little utility because it doesn't reflect the uncertainty manifested by the magnitude of the difference between the estimate and the true value. Statistical intervals can be used for this purpose. A discussion of statistical intervals can be found in *Basic Statistical Principles and Uncertainty, Statistical Intervals*. Interval estimates provide additional details that may be useful for risk based decision making.

Distributional estimates are used in Bayesian analysis to define expectations when the population parameter is viewed as a random variable. In particular, posterior distributions formed by combining prior and sample information are used to assign probabilities that the unknown parameter will fall in a given range. *Appendix 5: Bayesian Inference* describes the utility of distributional estimates in more detail.

A statistical paradigm used to express the objective of an inferential study is a statistical hypothesis test. A hypothesis test is expressed as a pair of statements called the null hypothesis (H_0) and the alternative hypothesis (H_a). Both are expressed concerning some unknown population parameter. Population parameters are often denoted with Greek letters. The Greek letter theta (θ) will be used for illustration. A two-sided hypothesis test can be written as

$$H_0: \theta = \theta_0$$
$$H_a: \theta \neq \theta_0 \qquad (6)$$

where θ_0 represents the hypothesized value for θ. The alternative hypothesis is sometimes called the research hypothesis because it represents the objective of the study. As an example, consider the true slope of a linear model representing the average change in the purity of a compound over time. Traditionally, this parameter is represented with the Greek letter beta (β). An investigator intends to determine if there is evidence that the average change in purity is a function of time. That is, if it can be shown that the true value of the slope is non-zero. Accordingly, *equation (6)* is written as

$$H_0: \beta = 0$$
$$H_a: \beta \neq 0 \qquad (7)$$

It should be noted that this is called a two-sided hypothesis because the direction of the difference is unspecified. This would be the case if the study sought to determine either a positive change (increase in purity) or a negative change (decrease in purity). But this is unlikely to be the desired objective of the study. It's more plausible that the study would strictly seek to determine if there is evidence that average purity decreases over time. This would be expressed as a one-sided hypothesis test as follows

$$H_0: \beta \geq 0$$
$$H_a: \beta < 0 \qquad (8)$$

The choice of two-sided or one-sided hypothesis test should be made when formulating the study objective, and prior to design and execution of the study. It should be based on a plausible scientific objective and should never be decided on the basis of the study results. Examples of two-sided and one-sided hypothesis tests will be given in *Comparison of Analytical Procedures*.

An additional consideration in formulating a study objective is the use of equivalence or noninferiority testing. These procedures require that the investigator formulate their hypotheses with a scientifically or practically meaningful objective. These will be illustrated in *Comparison of Analytical Procedures* and is discussed in detail in *Appendix 3: Equivalence and Noninferiority Testing*.

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Study Design

Study design should ensure an acceptable level of uncertainty in an estimation study or an acceptable risk for drawing the wrong conclusion in a test of inference. This can be managed through use of statistical design tools, including blocking and replication. As discussed previously, the design should also consider strategic selection of samples and study conditions which are associated with experiences in normal practice.

DESIGN OF AN ESTIMATION STUDY

The design of an estimation study may use sufficient replication (sample size) and blocking to ensure desired control of the uncertainty in the result. To illustrate, consider estimation of a mean based on a simple random sample of n units from a study population. The half width of the confidence interval (also called the margin of error) in *equation (5)* in *Basic Statistical Principles and Uncertainty* represents the uncertainty in the estimation of the mean. In planning the study, the margin of error can be defined to be no greater than a maximum allowable value H. Selecting the confidence level, $(1 - \alpha)$, and providing a preliminary estimate for the standard deviation (S), one can solve for a required sample size using the equation

$$t_{1-\alpha/2:n-1} \times \frac{S}{\sqrt{n}} \leq H$$

$$n \geq \frac{t^2_{1-\alpha/2:n-1} \times S^2}{H^2} \qquad (9)$$

Since the degrees of freedom of the t-value are a function of n, one must either solve *equation (9)* iteratively, or use an approximation by replacing the t-value with the associated Z-value. Preliminary estimates for S are obtained from similar studies or through the advice of subject matter experts. Scale of the data (e.g., transformed or original scale) should be defined prior to obtaining the preliminary estimate of the standard deviation or defining H (see *Appendix 2: Models and Data Considerations* for more on data transformation).

DESIGN OF AN INFERENTIAL STUDY

The design of an inferential study is based on controlling the risks of drawing the wrong conclusion. Following the paradigm of a hypothesis test, these risks are illustrated in *Table 1* and *Table 2*.

Table 1. Conclusions in a statistical test

	If H_0 is true	If H_0 is false
Reject H_0	Wrong conclusion (Type I error)	Correct conclusion
Do not reject H_0	Correct conclusion	Wrong conclusion (Type II error)

Table 2. Probabilities of a wrong conclusion

Wrong Conclusion	Probability of Occurrence
Type I error	α (called the level of significance)
Type II error	β ($1 - \beta$ is called the power)

It is important to determine the required sample size to control the Type I error (α) and Type II error (β) simultaneously. Formulas for sample sizes supporting an inferential study that depend on selected values of (α) and (β) are available in many textbooks and software packages. These formulas become more complex when the design includes blocking or experimental factors such as analyst or day. Computer simulation is a useful tool in these more complex situations, and support of a statistician can be useful.

While replication is an effective strategy for reducing the impact of random variability on uncertainty and risk, blocking can be used to remove known sources of variability. For example, in a study to compare two analytical procedures, each procedure might be used to measure each sample unit of material. This results in the removal of the variability between sample units of material, which provides a reduced error term used to compare differences between the two procedures. By reducing the error term in this manner, the power of the experiment is increased for a fixed number of sample units. A numerical example is provided in *Comparison of Analytical Procedures*.

Study Conduct

It is important to avoid introducing systematic error or bias into the study results. Bias can be introduced through unintentional changes in experimental conditions, due to either known or unknown factors. Effective sampling and randomization are important considerations in mitigating the impact of bias. Sampling is performed after the study has been designed and constitutes the selection of test articles within the structure of the design. How to attain such a sample depends entirely on the question that is to be answered by the data. When possible, use of a random process is considered the most appropriate way of selecting samples.

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The most straightforward type of random sampling is called simple random sampling. However, sometimes this method of selecting a random sample is not desirable because it cannot guarantee equal representation across study factors. The design of a study to release manufactured lots might incorporate factors such as selected times, locations, or parallel manufacturing streams (e.g., multiple filling lines). In this case a stratified sample whereby units are randomly selected from within each factor can be utilized. Regardless of the reason for taking a sample, a sampling plan should be established to provide details on how the sample is to be obtained to ensure that it is representative of the entirety of the population of interest.

Randomization should not be restricted to sampling. Study samples should be strategically entered into an analytical procedure using randomization, while blocking can be utilized to avoid confounding of the study objective with assay related factors.

Sometimes it's impossible to utilize sampling plans which are random or systematic in nature. This is especially true when the population is infinite. In this case representativeness is addressed through study design including blocking, where factors which are known to be the key structural components of the population are used to represent the infinite population.

The optimal sampling and analytical testing strategy will depend on knowledge of the manufacturing, analytical measurement, and/or study related processes. In the case of sampling to measure a property of a manufactured lot, it is likely that the sampling will include some element of random selection. There should be sufficient samples collected for the original analysis, subsequent verification analyses, and other supporting analyses. In the case of sampling to address a more complex study, representativeness should be addressed through strategic design. It is recommended that the subject matter expert work with a statistician to help select the most appropriate sampling plan and design for the specified objective.

An additional consideration in the conduct of a study is data recording. Many institutions store data in a Laboratory Information Management System (LIMS). That data may be entered to the number of significant digits (decimals) of the reportable value for the test procedure. While this practice is appropriate for the purpose of reporting test data (such as in a Certificate of Analysis or in a regulatory dossier), it is inappropriate for data which may be used for subsequent analysis. This is noted in ASTM E29 where it is stated "As far as is practicable with the calculating device or form used, carry out calculations with the test data exactly and round only the final result". Rounding intermediate calculated results contributes to the overall error in the final result. More on rounding is included in *General Notices, 7.20 Rounding Rules* and in *Appendix 2: Models and Data Considerations*.

Study Analysis

The culmination of a study is a statistical analysis of the data, and a decision in the case of an inferential study. Simple summaries such as group averages and appropriate measures of variability, as well as plots of the data and summary results facilitate the analysis and communication of the study results and decision. Summaries should be supplemented with confidence intervals or bounds, which express the uncertainty in the summary result (see *Basic Statistical Principles and Uncertainty*). Transformations based on either scientific information or empirical evidence can be considered, and screening for outlying values and subsequent investigations completed (see *Appendix 2: Models and Data Considerations*).

Many common statistical analysis tools are found in calculation programs such as spreadsheets and instrument software. Software which is dedicated to statistical analysis and modeling contain additional tools to evaluate assumptions associated with the analysis tools, such as normality, homogeneity of variance, and independence. Those with limited or no statistical training should consult a statistician throughout the process of conducting a study, including study design and analysis. Their statistical skills complement the laboratory skills in ensuring appropriate study design, analysis, and decisions.

The study considerations outlined in this section will be illustrated hereafter.

5. COMPARISON OF ANALYTICAL PROCEDURES

It is often necessary to compare two analytical procedures to determine if differences in accuracy and precision are less than an amount deemed practically important. For example, General Notices 6.30 describes the need to produce comparable results to the compendial method. Transfer of analytical procedures as described in *Transfer of Analytical Procedures* ⟨1224⟩ allows for comparative testing as an acceptable process. A change in a procedure includes a change in technology, a change in laboratory (called transfer), or a change in the reference standard in the procedure.

For purposes of this section, the terms old procedure and new procedure are used to represent a procedure before and after a change. Procedures with differences less than the practically important criterion are said to be equivalent or better (see *Appendix 3: Equivalence and Noninferiority Testing*). This section follows the outline described in *Study Considerations* highlighting the scientific method of (1) study objective, (2) study design, (3) study conduct, and (4) study analysis.

Study Objective of a Procedure Comparison

The study objective of a procedure comparison is to demonstrate that a new procedure performs equivalent to or better than an old procedure. There are two conceptual study populations: All future measurements made with the old procedure on a particular process, and all future measurements made with the new procedure on the same process. Each procedure is described in terms of the mean and standard deviation of the population of measurements. The mean and standard deviation of the reportable value of the new procedure are denoted by the Greek symbols μ_N and σ_N respectively. The subscript N denotes the "new" procedure population. The mean and standard deviation of measurements using the "old" procedure are denoted μ_O and σ_O respectively. These means and standard deviations are unknown, but conclusions concerning their potential equivalence or noninferiority (the new procedure is not inferior to the old procedure) are informed by estimates resulting from the experiment. Characteristics for comparison are most generally accuracy and precision across the range of the assay, and

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across conditions experienced during long term routine analysis. A risk analysis should be performed to identify such conditions. Discussion of accuracy and precision are found in ⟨1225⟩.

ACCURACY

To compare accuracy of two procedures, one compares the procedure means. In particular, accuracy is compared using the absolute value of the true difference in means,

$$|\mu_D| = |\mu_N - \mu_O|. \qquad (10)$$

The objective of such a study is to demonstrate that $|\mu_D|$ is less than a value deemed to be practically important, d. As an example, d may represent a numerical value that is small enough so that an increase in bias of this magnitude does not negatively impact decisions concerning lot disposition (i.e., conformance to specifications). The hypotheses used in an equivalence test are

$$H_0: |\mu_D| \geq d$$
$$H_a: |\mu_D| < d \qquad (11)$$

(see *Appendix 3: Equivalence and Noninferiority Testing*).

Probably the most difficult aspect of conducting an equivalence test is determination of d. Typically, d is determined in partnership between the analytical chemist and the statistician based on combined manufacturing and scientific knowledge. Definitions of d vary across companies based on differing risk profiles and experience. In some cases there exists a large amount of legacy data that may inform the decision, while in other cases there may be only limited data. An example where d is based on requirements of a manufacturing process follows in the section *Determination of d and k*.

PRECISION

To compare precision of two procedures, one compares the procedure standard deviations. Whereas a comparison of means involves a difference, a comparison of standard deviations involves the ratio

$$\frac{\sigma_N}{\sigma_O} \qquad (12)$$

The study objective is to demonstrate that the ratio in *equation (12)* is less than a practically important value k. The noninferiority hypotheses are

$$H_0: \frac{\sigma_N}{\sigma_O} \geq k$$
$$H_a: \frac{\sigma_N}{\sigma_O} < k \qquad (13)$$

(see *Appendix 3: Equivalence and Noninferiority Testing*). The selection of k should be in alignment with the selection of d for the accuracy assessment. This process is demonstrated in the following section.

DETERMINATION OF *d* AND *k*

Values of d and k for the tests of accuracy and precision should be internally consistent. To demonstrate, consider a case where historical measurements using an old procedure for a monitored process have a process mean of $\mu_O = 100$ units and a combined process and analytical variance of $\sigma_L^2 + \sigma_O^2 = 0.80$ where σ_L^2 represents lot-to-lot variability of the manufacturing process. Historic measurements of a reference standard provide the estimate $\sigma_O^2 = 0.16$ so that the assumed value of the lot variance is $\sigma_L^2 = 0.80 - 0.16 = 0.64$. The process specifications are the lower specification limit $LSL = 96$ units and the upper specification limit $USL = 104$ units. The same manufacturing process measured with the new procedure can be represented as having mean $\mu_N = \mu_O + d$ and total process and analytical variance $\sigma_L^2 + \sigma_N^2 = \sigma_L^2 + k^2\sigma_O^2$.

Kringle et al. (2001) recommend selecting values of d and k consistent with a rule that states the proportion of product that falls outside of specification (OOS) when measured with the new procedure is acceptable. Table 3 reports the OOS rate when the process is in control and measured with the new procedure for several values of d and k. (Since the specifications are symmetric around μ_O, negative values of d provide the same OOS rates as the positive values shown in the *Table 3*).

Table 3. OOS rate with new procedure for values of *d* and *k*

d	*k*=1	*k*=1.5	*k*=2
0	0.001%	0.01%	0.04%
1	0.04%	0.14%	0.40%

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Table 3. OOS rate with new procedure for values of *d* and *k* *(continued)*

d	*k*=1	*k*=1.5	*k*=2
2	1.27%	2.28%	3.85%

Table 3 assumes the process is normal and the probability in any cell is given by the equation

$$Pr(\text{OOS}) = 1 - Pr(96 \le \text{Sampled process value} \le 104)$$
$$= 1 - \Phi\left(\frac{104 - (100 + d)}{\sqrt{0.64 + k^2 \times 0.16}}\right) - \Phi\left(\frac{96 - (100 + d)}{\sqrt{0.64 + k^2 \times 0.16}}\right) \qquad (14)$$

where $\Phi(\bullet)$ represents the cumulative probability function of the standard normal distribution. Suppose that the risk profile allows an OOS rate no greater than 1.0%. Based on *Table 3*, a consistent set of criteria are d=1 and k=2.

Study Design of a Procedure Comparison

The study design for comparing the old and new analytical procedures is comprised of the selection of test materials, experimental design, and sample size determination (the so-called power calculation). Results for two scenarios are provided in this section. The first scenario considers samples from homogeneous test material, and the second scenario considers test material with variation across sample units.

SCENARIO 1: HOMOGENEOUS TEST MATERIAL

In this scenario, test samples of homogeneous material are selected and measured using one of the procedures on each test sample. There are n_O samples measured with the old procedure and n_N samples measured with the new procedure. It is recommended to design the study so that $n_O = n_N$. *Table 4* presents this design which is referred to as an independent two-sample design.

Table 4. Independent two-sample design

Sample ID	New Procedure	Old Procedure
1	Y_{N1}	
2	Y_{N2}	
⋮	⋮	
n_N	Y_{Nn_N}	
$n_N + 1$		Y_{O1}
$n_N + 2$		Y_{O2}
⋮		⋮
$n_N + n_O$		Y_{On_O}
Sample Mean	$\bar{Y}_N = \frac{\sum_{j=1}^{n_N} Y_{Nj}}{n_N}$	$\bar{Y}_O = \frac{\sum_{j=1}^{n_O} Y_{Oj}}{n_O}$
Sample Variance	$s_N^2 = \frac{\sum_{j=1}^{n_N} (Y_{Nj} - \bar{Y}_N)^2}{n_N - 1}$	$s_O^2 = \frac{\sum_{j=1}^{n_O} (Y_{Oj} - \bar{Y}_O)^2}{n_O - 1}$

For the comparison of means the estimator of interest is the difference of sample means, $\bar{Y}_N - \bar{Y}_O$ which has variance

$$\text{Var}(\bar{Y}_N - \bar{Y}_O) = \sigma_N^2/n_N + \sigma_O^2/n_o \qquad (15)$$

Power calculations are needed to ensure the sample size is great enough to find evidence that H_a is true when such is the case. For testing the equivalence hypotheses in *equation (11)* assuming $\sigma_N = \sigma_O$, Bristol (1993) recommends the sample size formula

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$$n_N = n_O = 2 \times \left(\frac{\left(Z_{1-\alpha} + Z_{1-\beta}\right) \times \sigma_O}{d - |\mu_D|} \right)^2 + 1 \qquad (16)$$

where $Z_{1-\alpha}$ and $Z_{1-\beta}$ are standard normal percentiles with area $1-\alpha$ and $1-\beta$ respectively, to the left. The Type I error rate is α and the Type II error rate is β. To make this calculation consistent with the case where σ_N can be as great as $k\sigma_O$, a recommended modification is

$$n_N = n_O = (1 + k)^2 \times \left(\frac{\left(Z_{1-\alpha} + Z_{1-\beta}\right) \times \sigma_O}{d - |\mu_D|} \right)^2 + 1 \qquad (17)$$

The information provided earlier to select d=1 and k=2 is now used to determine sample size for the study. For the test of equivalence of means, it is desired to have a high probability of passing when the two means are equal, that is when $\mu_D = 0$. So setting $\beta = 0.10$ and $\alpha = 0.05$ with $\sigma_O = \sqrt{0.16} = 0.4$, the required sample size for both the new and old procedures using *equation (17)* is

$$n_N = n_O = (1 + 2)^2 \times \left(\frac{(1.645 + 1.282) \times 0.4}{1 - 0} \right)^2 + 1 = 7.9 \qquad (18)$$

which is rounded up to 8 for each procedure (for 16 total test samples).

To test the noninferiority hypotheses in *equation (13)*, it is desired to have a high power when $\sigma_N = \sigma_O$. The required sample size is obtained by solving for n_N and n_O iteratively using the equation

$$1 - \beta = Pr\left(F < \frac{\sigma_O^2 k^2}{\sigma_N^2} \times F_{\alpha: n_N - 1, n_O - 1} \right) \qquad (19)$$

where F is a random variable following the F-distribution with degrees of freedom $n_N - 1$ and $n_O - 1$. As noted earlier, it is recommended that $n_N = n_O$ and the sample size is the greater of the requirements from *equations (17)* and *(19)*. *Table 5* reports the power for sample size combinations using previous information when $\alpha = 0.05$ and $\sigma_N = \sigma_O = 0.4$.

Table 5. Power calculation for noninferiority test with $\alpha = 0.05$

n_N	n_O	$\frac{\sigma_O^2 k^2}{\sigma_N^2}$	$F_{\alpha: n_N - 1, n_O - 1}$	$\frac{\sigma_O^2 k^2}{\sigma_N^2} \times F_{\alpha: n_N - 1, n_O - 1}$	Power when $\sigma_N = \sigma_O$
8	8	4	0.264	1.056	0.528
14	14	4	0.388	1.552	0.781
15	15	4	0.403	1.610	0.808
19	19	4	0.451	1.804	0.890
20	20	4	0.461	1.845	0.904

From *Table 5* it is seen that the sample of size 8 required for the test of equivalence of means does not provide acceptable power for the noninferiority test (Power = 0.528). This is because estimates of standard deviations have greater uncertainty than estimates of means. Practicality often dictates that one select a greater value for β in a test of noninferiority than in a test for equivalence of means. In the present example, β is selected as 0.20 and a sample size of 15 per procedure (30 test samples in total) is selected for the design.

When a comparison is made between laboratories (as during procedure transfer) it's important to keep in mind that in order to be representative of future testing, the study design should include factors which have significant impact on the long term performance of the procedure. As noted previously, this may include analyst, but may also require that multiple instruments and batches of key reagents be included in the design. These may be nested or crossed. Failing to do so may underestimate the variability or confound the effects of some factors with the difference between labs. In general factors such as analysts where levels are unique within each laboratory might be nested within each lab, while factors such as reagent lots which might be routinely shared across laboratories could be crossed with laboratory. As such, the estimates of variability used in these equations should be representative of the variability induced by these factors. The best estimates of variability come from data collected on samples tested across a broad period of time, such as stability samples and an assay control. More considerations of this nature are described in ⟨1210⟩.

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SCENARIO 2: VARIATION ACROSS TEST SAMPLES

It is often desirable to compare procedures across manufactured lots or use different manufactured levels of an analyte. This is important if the study objective is to ensure the range of the procedure in the new laboratory, or when the procedure is intended to measure degraded samples. This selection of test material introduces a new source of variation to *Scenario 1* that must be considered during the study design in order to most efficiently compare the two procedures.

The recommended design in *Scenario 2* is a paired design in which each test sample is measured independently by both procedures, instead of having each test sample randomly measured by only one procedure as in *Scenario 1*. The term "Test Sample" is referred to as a blocking factor because observations within the same block are differenced (see *Study Considerations*). This has the effect of removing the variation across test samples from the analysis. *Table 6* presents a schematic illustration of the paired design using n test samples.

Table 6. Paired design

Test Sample	New Procedure	Old Procedure	Difference
1	$Y_{N,1}$	$Y_{O,1}$	$D_1 = Y_{N,1} - Y_{O,1}$
2	$Y_{N,2}$	$Y_{O,2}$	$D_2 = Y_{N,2} - Y_{O,2}$
⋮	⋮	⋮	⋮
n	$Y_{N,n}$	$Y_{O,n}$	$D_n = Y_{N,n} - Y_{O,n}$
Sample Mean	$\overline{Y}_N$	$\overline{Y}_O$	$\overline{D} = \overline{Y}_N - \overline{Y}_O = \frac{\sum_{j=1}^{n} D_j}{n}$
Sample Variance	NA	NA	$S_D^2 = \frac{\sum_{j=1}^{n} (D_j - \overline{D})^2}{n-1}$

Using the paired design with n lots, the variance of $\overline{D}$ is $(\sigma_N^2 + \sigma_O^2)/n$ because the variability due to lots disappears when results on the same lot are differenced. The unbiased estimator of $\sigma_N^2 + \sigma_O^2$ is S_D^2.

The sample size formula for satisfying the mean test requirements for a paired design adjusting for the fact that σ_N^2 can be as great as $k^2\sigma_O^2$ is

$$n = \left(\frac{(Z_{1-\alpha} + Z_{1-\beta}) \times \sqrt{\sigma_N^2 + \sigma_O^2}}{d - |\mu_D|} \right)^2 + 1$$

$$= \left(\frac{(Z_{1-\alpha} + Z_{1-\beta}) \times \sqrt{k^2\sigma_O^2 + \sigma_O^2}}{d - |\mu_D|} \right)^2 + 1$$

$$= (1 + k^2) \times \left(\frac{(Z_{1-\alpha} + Z_{1-\beta}) \times \sigma_O}{d - |\mu_D|} \right)^2 + 1 \qquad (20)$$

which is the same formula shown in *equation (17)*.

Using the same planning data from Scenario 1, the test for equivalence of means with $\beta = 0.10$ when $\mu_D = 0$ and $\alpha = 0.05$ is as before

$$n = (1 + 2^2) \times \left(\frac{(1.645 + 1.282) \times 0.4}{1 - 0} \right)^2 + 1 = 7.9 \qquad (21)$$

which is rounded up to 8 test samples (which are each measured once by each procedure). When using a paired design for the test of non-inferiority, the ability to find a good estimate of σ_O^2 is critical. Good estimates of σ_O^2 are often available from previous method validation studies or repeated measurements of an assay control. If no such estimate exists, it is necessary to modify the design in *Table 6* and record two independent measurements with each procedure on each test sample. Independent estimates of both σ_O^2 and σ_N^2 can then be computed from the differences of the two paired values as shown in the section *Study Analysis of a Procedure Comparison* that follows.

If a good estimate for σ_O^2 is available, the required sample size for the noninferiority test is derived iteratively from the equation

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$$1-\beta = Pr\left(W < \frac{\left(k^2+1\right)\sigma_O^2 \times \chi^2_{\alpha:n-1}}{\sigma_N^2+\sigma_O^2}\right) \qquad (22)$$

where W is a chi-squared random variable with $n-1$ degrees of freedom.
Table 7 reports the power for sample size combinations when $\alpha = 0.05$ and $\sigma_N = \sigma_O = 0.4$.

Table 7. Power calculation for noninferiority test with $\alpha = 0.05$

n	$\frac{\left(k^2+1\right)\sigma_O^2}{\sigma_N^2+\sigma_O^2}$	$\chi^2_{\alpha:n-1}$	$\frac{\left(k^2+1\right)\sigma_O^2}{\sigma_N^2+\sigma_O^2} \times \chi^2_{\alpha:n-1}$	Power when $\sigma_N = \sigma_O$
8	2.5	2.167	5.418	0.391
17	2.5	7.962	19.904	0.775
18	2.5	8.672	21.679	0.803
22	2.5	11.591	28.978	0.885
23	2.5	12.338	30.845	0.901

To obtain a power of 0.80 when the two standard deviations are equal, a sample of 18 test samples is required. Note that each test sample need not be unique. For example, if samples are being selected from three lots of product, one could select six test samples from each lot.

Study Conduct of a Procedure Comparison

When conducting the study, it is important to observe the random assignment of test samples to procedures in *Scenario 1* in order to guard against possible bias. If repeated measurements are used in *Scenario 2* to provide individual estimates of σ_O^2 and σ_N^2, then independent measurements are needed. This will require independent preparations for each portion of the test sample.

Study Analysis of a Procedure Comparison

Two examples are provided to demonstrate the described formulas. Data in the examples were simulated from a population where $\mu_N = \mu_O = 100$ and $\sigma_N^2 = \sigma_O^2$. These values were selected to demonstrate the computed sample sizes are sufficient under the assumed conditions.

SCENARIO 1: HOMOGENEOUS TEST MATERIAL

Table 8 reports a sample data set with $n_N = n_O = 15$.

Table 8. Data from simulated two-sample independent design

Procedure	Sample Mean	Sample Variance
New	$\bar{Y}_N = 100.08$	$S_N^2 = 0.214$
Old	$\bar{Y}_O = 99.85$	$S_O^2 = 0.159$

Accuracy is tested using the hypotheses in *equation (11)* by constructing a $100(1-2\alpha)\%$ confidence interval on μ_D using the equation

$$\bar{Y}_N - \bar{Y}_O \pm t_{1-a:df}\sqrt{\frac{S_N^2}{\eta_N}+\frac{S_O^2}{\eta_O}}$$

$$df = \frac{\left(\frac{S_N^2}{n_N}+\frac{S_O^2}{n_O}\right)^2}{\frac{S_N^4}{n_N^2\left(n_N-1\right)}+\frac{S_O^4}{n_O^2\left(n_O-1\right)}} \qquad (23)$$

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where $t_{1-\alpha:df}$ is a quantile from a central *t*-distribution with area $1-\alpha$ to the left and degrees of freedom *df*. The null hypothesis in *equation (11)* is rejected, and equivalence demonstrated if the entire confidence interval computed from *equation (23)* falls in the range from $-d$ to $+d$. This is the TOST described in *Appendix 3: Equivalence and Noninferiority Testing* and has a Type I error rate of α. With some software packages such as Excel, non-integer *df* values are not accepted when determining the *t*-value. In this case, simply round to the nearest integer.

The 90% two-sided confidence interval that provides a Type I error rate of 0.05 computed from *equation (23)* is

$$df = \frac{\left(\frac{0.214}{15} + \frac{0.159}{15}\right)^2}{\frac{0.214^2}{15^2(15-1)} + \frac{0.159^2}{15^2(15-1)}} = 27.4 = 27 \text{ (rounded)}$$

$$\bar{Y}_N - \bar{Y}_O \pm t_{1-\alpha:df}\sqrt{\frac{S_N^2}{n_N} + \frac{S_O^2}{n_O}}$$

$$100.08 - 99.85 \pm 1.703\sqrt{\frac{0.214}{15} + \frac{0.159}{15}}[-0.04\ ;0.50]. \qquad (24)$$

Since the computed confidence interval falls entirely in the range between −1 and +1 (i.e., $-d$ to $+d$) equivalence of means has been demonstrated.

Precision is tested using the hypotheses in *equation (13)* by constructing a $100(1-\alpha)\%$ one-sided upper confidence bound on the ratio σ_N/σ_O using the formula

$$\frac{S_N}{S_O}\sqrt{\frac{1}{F_{\alpha,n_N-1,n_O-1}}} \qquad (25)$$

where $F_{\alpha,n_N-1.n_O-1}$ is the *F*-quantile with area α to the left and degrees of freedom $n_N - 1$ and n_O. If the upper bound computed with *equation (25)* is less than *k*, the null hypothesis is rejected and one concludes noninferiority of the standard deviation of the new procedure. This test has a Type I error rate of α.

The 95% upper bound on σ_N/σ_O computed from *equation (25)* is

$$U = \frac{\sqrt{0.214}}{\sqrt{0.159}}\sqrt{\frac{1}{0.402}} = 1.83 \qquad (26)$$

Since this upper bound is less than $k = 2$, noninferiority of the standard deviation of the new procedure has been demonstrated.

SCENARIO 2: VARIATION ACROSS TEST SAMPLES

Table 9 provides summary results for 18 test samples in a paired design with $\bar{D} = \bar{Y}_N - \bar{Y}_O$.

Table 9. Data from simulated paired design with n=18

Sample Mean	Sample Variance
$\bar{D} = 0.39$	$S_D^2 = 0.350$

The 90% confidence interval on the difference in means for a paired design used to test equivalence of means with the data from *Table 9* is

$$\bar{D} \pm t_{0.95;n-1}\sqrt{\frac{S_D^2}{n}}$$

$$0.39 \pm 1.74\sqrt{\frac{0.350}{18}}$$

$$[0.15 \text{ to } 0.63] \qquad (27)$$

Since the computed confidence interval falls entirely in the range between −1 and +1 equivalence of means has been demonstrated.

The noninferiority hypotheses in *equation (13)* can be tested by constructing a $100(1-\alpha)\%$ upper confidence bound on σ_N/σ_O using the formula

$$\sqrt{\frac{(n-1)S_D^2}{\sigma_O^2 \times \chi^2_{\alpha:n-1}} - 1} \qquad (28)$$

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where $\chi^2_{\alpha;n-1}$ is a percentile from the chi-squared distribution with area α to the left and degrees of freedom $n-1$. If this upper bound is less than k, the null hypothesis is rejected and noninferiority has been demonstrated.

From historical data used to plan the sample size, a good estimate of the old procedure variance is $\sigma_O^2 = 0.16$. Using the confidence bound in *equation (28)*, the 95% upper confidence bound on σ_N/σ_O is

$$U = \sqrt{\frac{(18-1)0.350}{0.16 \times 8.67} - 1}$$
$$U = 1.81 \qquad (29)$$

Since this upper bound is less than k=2, noninferiority of the standard deviation of the new procedure has been demonstrated.

If a good estimate of σ_O^2 is not available, the design requires replicate measures for each procedure on each test sample. Independent estimates of the analytical variances are computed using the formulas

$$S_{DN}^2 = \frac{\sum_{j=1}^{n}\left(\frac{Y_{jN1} - Y_{jN2}}{\sqrt{2}} - \bar{D}_N\right)^2}{n-1}$$
$$S_{DO}^2 = \frac{\sum_{j=1}^{n}\left(\frac{Y_{jO1} - Y_{jO2}}{\sqrt{2}} - \bar{D}_O\right)^2}{n-1} \qquad (30)$$

where Y_{jN1} is the first measurement on test sample j with method N, Y_{jN2} is the second measurement on test sample j with method N, Y_{jO1} is the first measurement on test sample j with method O, and Y_{jO2} is the second measurement on test sample j with method O. The resulting $100(1-\alpha)$% one-sided upper confidence bound on the ratio σ_N/σ_O is

$$\frac{S_{DN}}{S_{DO}}\sqrt{\frac{1}{F_{\alpha,n-1,n-1}}} \qquad (31)$$

where $F_{\alpha,n-1,n-1}$ is the F-quantile with area α to the left and degrees of freedom $n-1$ and $n-1$, and n is the number of test samples (each with four independent measures). If this formulation is needed, then define $D_j = ((Y_{jN1} + Y_{jN2}) - (Y_{jO1} + Y_{jO2})/\sqrt{2}$ in the test for mean equivalence.

APPENDIX 1: CONTROL CHARTS

Control charts are used in the pharmaceutical industry to monitor the performance of manufacturing processes and analytical procedures. Using the vernacular of the scientific method, control charts are a tool to study these process populations, requiring a carefully developed objective, a strategic design, plans for implementation, and appropriate analysis. This appendix will discuss and illustrate the design and analysis of various control chart tools, as well as provide rules which are commonly used to make decisions.

Through its lifecycle a process or a procedure can be influenced by known changes or unforeseen variability. For a manufacturing process this might impact the quality of the product or indicate the need to take action. For an analytical procedure which is routinely used to aid decision-making, this might increase the risk of drawing the wrong conclusion from a study or likewise indicate the need for action. Thus, it is important to continuously verify performance and provide ongoing assurance of a state of control. To this end, data from a manufacturing process or that relate to procedure performance are collected and analyzed. For a manufacturing process these may include process parameters and test results on manufactured materials. For an analytical procedure they can include analytical results for controls, standards used during the analysis, and system suitability data. It's important to note that the control samples are used to monitor the performance of the procedure and are not an indicator of the product performance or characteristics (FDA ISO 17025). For purposes of this appendix the term process will be used to refer to both a manufacturing process and an analytical procedure.

Although various trending methods exist, control charts are one of the most simple and effective graphical tools for such analysis. There are many types of control charts including the following:

- Individual (I) chart for plotting individual values over time,
- X-bar chart for plotting sample means over time,
- Range (R) chart for plotting sample ranges over time,
- Moving range (MR) chart for plotting moving ranges over time,
- S-chart for plotting sample standard deviations over time, and
- Exponentially weighted moving average (EWMA) and cumulative sum (CUSUM) charts which are used when small shifts in the mean of the procedure are of interest.

A typical control chart consists of a centerline and lower and upper control limits. The centerline represents the center of the distribution of a variable measured in the process. The two control limits are determined such that if the process performs as intended, nearly all results will fall within the two limits. Observations outside the limits or points within the limits that indicate a

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systematic or non-random pattern are indicative of a potential performance issue. Non-systematic patterns have been defined by WECO (which stands for Western Electric Company) and Nelson (1984) that can be used in evaluating a control chart. Historical data (the "control data") are typically used to obtain the centerline and lower and upper control limits. The control chart provides a visual means for identifying shifts, trends, and variability indicative of potential performance issues. A clarifying example is presented in the next section based on the Individual or I-chart.

Shewhart I-Chart

To develop a control chart for individual observations, it is customary to set control limits at

$$\text{Process Mean} \pm 3 \times \text{Process Standard Deviation} \quad (32)$$

These limits are based on assuming the process data follow a normal probability distribution and that a range of 3 standard deviations about the mean contains roughly 99.7% of all the data. Given a sample of $Y_1, Y_2, \ldots, Y_n$ observations from a controlled process, the process mean (average) is estimated using the formula

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} \quad (33)$$

The standard deviation can be estimated in a couple of ways, but for an I-chart, best practice is to base the estimate on the moving range statistic (MR). This estimator considers the "short term" variability of the process and guards against limits that are too wide if an unexpected trend exists in the data. Specifically, the MR represents the average difference of successive observations and is defined as

$$\overline{MR} = \frac{\sum_{i=2}^{n} |Y_i - Y_{i-1}|}{n-1} \quad (34)$$

and the estimator for the process standard deviation is

$$\frac{\overline{MR}}{d_2} \quad (35)$$

where d_2 is a constant that depends on the number of observations associated with the moving range calculation (m). In *equation (34)* $m = 2$ since the range is based on adjacent observations. The value of d_2 when $m = 2$ is 1.128. The upper control limit (UCL) and lower control limit (LCL) for the I-chart are then

$$LCL = \overline{Y} - 3 \times \frac{\overline{MR}}{d_2}$$

$$UCL = \overline{Y} + 3\frac{\overline{MR}}{d_2} \quad (36)$$

To demonstrate, consider a sample of 20 observations with $\overline{Y} = 31.2$ and $\overline{MR} = 2.18$. From *equation (36)* the computed control limits are

$$\text{LCL} = 31.2 - 3 \times \frac{2.18}{1.128} = 25.4$$

$$\text{UCL} = 31.2 + 3 \times \frac{2.18}{1.128} = 37.0 \quad (37)$$

The associated I-chart is shown in *Figure 1*.

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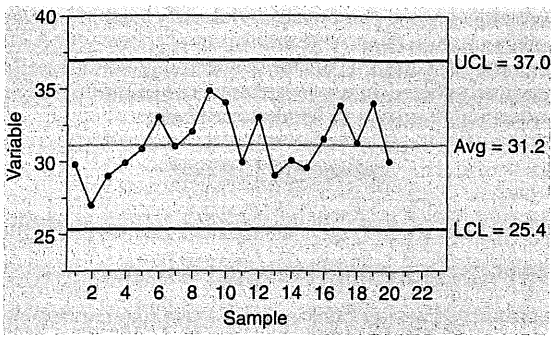

Figure 1. I-chart for example data set.

Detection of Out-Of-Control Results

After a control chart is constructed, out-of-control results are detected using either WECO or Nelson rules. The Nelson rules are provided in *Table 10*. The relevance of these rules depends on the type of control chart. All eight rules can be applied to an I-chart, and selection of the particular rules depends on the desired sensitivity of the control process.

Table 10. Nelson rules for detection of out-of-control results

Rule	Description	Indication in an I-chart
1	One point exceeds either the LCL or UCL.	One point is out of control
2	Nine points in a row on the same side of the center line	There is a mean shift in performance
3	Six points in a row steadily increasing or decreasing	A trend exists
4	14 points in a row alternating up and down	There is a negative correlation between neighboring points
5	Two out of three points on the same side of the mean and greater than two standard deviations away from the mean.	A possible increase in assay variability
6	Four out of five points on the same side of the mean and greater than one standard deviation away from the mean	A possible increase in assay variability
7	15 points in a row within one standard deviation of the mean	A possible decrease in assay variability
8	Eight points in a row on both sides of the mean with none within one standard deviation of the mean	Non-random sample

Figure 2 presents an I-chart for which a Rule 2 violation is observed because the last nine observations are all greater than the mean.

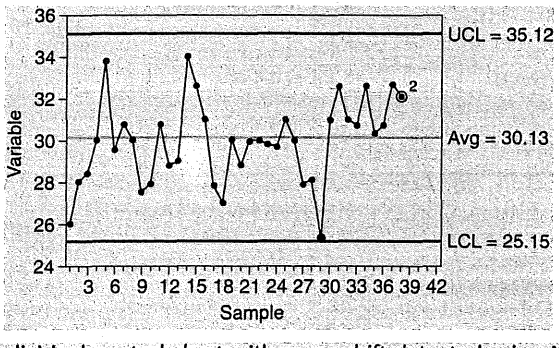

Figure 2. Individual control chart with mean shift detected using Nelson rule 2.

ASTM E2587 (2016), Montgomery (2012), and Wheeler (2012) provide references for numerous control charts and example applications.

APPENDIX 2: MODELS AND DATA CONSIDERATIONS

Statistical analysis involves models and assumptions associated with the reliability of fitting models to data. Models can be simple (e.g., a means model associated with a reportable value) or complicated (e.g., a nonlinear mixed effects model common in complex pharmaceutical settings). Assumptions monitored with residuals from the model fit include normality, constant

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variance, and independence. This appendix focuses on adequacy of models that are fit to analytical data, as well as data considerations such as significant digits, transformations, and outliers.

Models

In statistics, a model represents a functional description of some property(s) of a population. The term population refers to the set of all possible values of an attribute. A model parameter, also referred to as a population parameter, is the true but unknown value of a property, which is typically the subject of the statistical inquiry.

A means model characterizes the center of a univariate population, and can be written as

$$Y_i = \mu + E_i \qquad (38)$$

where Y_i is the ith observation in a sample of size n from the population, μ is a model parameter representing the population mean, and E_i the error. This error represents the effect of all factors that explain why the measured value is not always equal to μ. Such factors typically include lot-to-lot variation in product or analytical method error. The means model is the basis of statistical inquiries related to a population mean, usually estimated by the sample mean

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} \qquad (39)$$

with errors estimated by residuals $R_i = Y_i - \overline{Y}$.

Another familiar model is the simple linear regression model. This model characterizes the linear trend in the population mean with some covariate X_i (e.g., time or dose), and can be written as

$$Y_i = \alpha + \beta X_i + E_i \qquad (40)$$

where (X_i, Y_i) is the ith observation in a sample of size n from the bivariate population, the parameters α and β are the intercept and the slope, respectively, that defines the functional relationship and E_i the error. Note that μ in *model (38)* has been replaced with $\alpha + \beta X_i$ in *model (40)* to allow the mean to change as a function of X_i. The parameters α and β are estimated from sample data as was in *model (38)*.

More complex models might be nonlinear, can include qualitative factors (e.g., analysts in a validation), or might include covariables which are random rather than fixed values (e.g., another measurement Z_i made together with X_i).

Significant Digits

The number of digits used for calculations and the number of digits appearing in a reportable value should be considered separately. It's important to record and carry more digits during calculation than will be reported. It is a good practice to perform all statistical calculations with as many digits as practical. Rounding should be used only as a final step before reporting the result. Automation facilitates the acquisition of numerous digits, while databases should be designed to store data with enough digits in anticipation of further calculations from the data.

The number of digits reported can sensibly be based on the standard deviation of the reportable value. ASTM E29, USP *General Notices, 7.20 Rounding Rules*, and (*23*) provide guidance on rounding and determination of significant digits in a reported value.

Transformation

A transformation is a functional re-expression of a measurement in order to better represent a known scientific relationship or to satisfy the assumptions of a statistical model. Transformations can also be discovered empirically with a representative set of the data using residual plots. One particularly useful transformation with analytical data is the logarithmic (log) transformation described in the next section.

LOG TRANSFORMATION

Examples of transformations using scientific knowledge of the measurement system come from many biological systems. In particular, variation around the responses predicted by a means model is often proportional to the response. For these systems, it is useful to work with the log of the original response which will have nearly constant variance across the range of the response. The shape of the transformed distribution will also be more symmetric as shown in the lower panel of *Figure 3*. A log transformation can be conducted using any base including Napierian (base e), common (base 10), or base 2.

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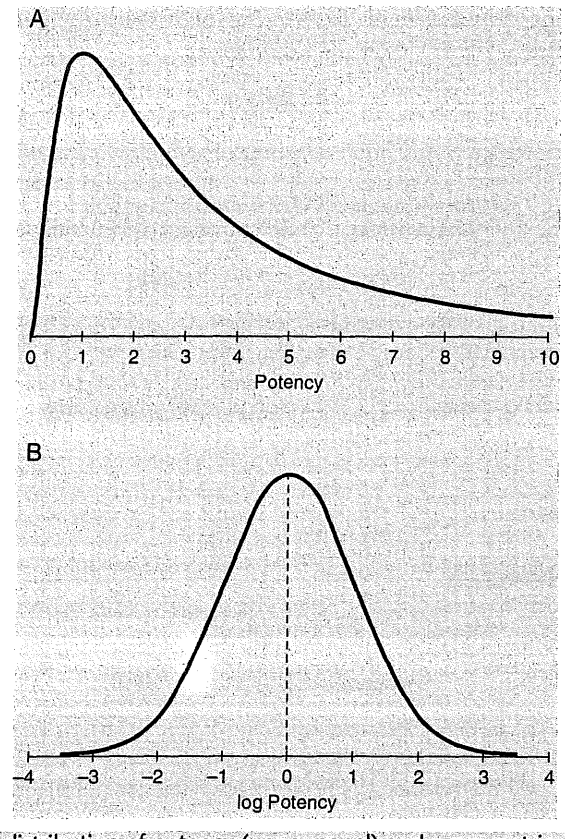

Figure 3. A skewed log-normal distribution of potency (upper panel) and a symmetric normal distribution of log potency (lower panel).

Another reason for using a log transformation is that it can change a nonlinear functional form in the original scale to something more easily modeled in the log scale. For example, a log transformation can be used to re-express a nonlinear first order kinetics model as a linear model.

Statistical measures associated with the center and the dispersion from a sample are described in *Basic Statistical Principles and Uncertainty*. These include the sample mean ($\overline{Y}$) the sample standard deviation (S). These measures are meaningful when the data are approximately normally distributed and free of outliers. These measures may not be as meaningful when the normal distribution is not a good description of the data. To demonstrate, the top distribution in *Figure 3* is skewed to the right. The greater values in the tail have the effect of pulling the mean to the right of where some would deem to be the "center" of the data. The lower distribution in *Figure 3* shows the log-transformed responses of the top distribution. The top distribution is called a log-normal distribution because the distribution of its log values is normal. Because of the symmetry of the normal curve, the sample mean and sample standard deviation are meaningful estimates of the center and dispersion of the transformed distribution.

The sample mean of log-transformed responses can be transformed back to the original scale. This back-transformation results in what is called the geometric mean (GM) on the original scale. More formally, let Y_i represent a measured response on the original scale and T_i the transformed value of Y_i. Then

$$T_i = \ln(Y_i)$$

$$\overline{T} = \frac{\sum_{i=1}^{n} T_i}{n}$$

$$\mathrm{GM} = \exp(\overline{T}) = \left(\prod_{i=1}^{n} Y_i\right)^{\frac{1}{n}} \qquad (41)$$

The standard deviation of log-transformed responses (S_T) can likewise be back-transformed as $\exp(S_T)$. This term is referred to as the geometric standard deviation (GSD) by Kirkwood (1979). That is,

$$\mathrm{GSD} = \exp(S_T) \qquad (42)$$

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Because S_T is non-negative, $GSD \geq 1$ and represents a fold-variation in the response scale. While a summary for arithmetically scaled responses can be written as ± S, this might be summarized as GM ×/÷ GSD, or GM/GSD to GM × GSD for geometrically scaled responses. If for example GSD = 1.25 and GM = 1.0, a range might be summarized as 1.0/1.25 = 0.80 to 1.0 × 1.25 = 1.25. It should be noted that this represents a 1-standard deviation range. A more appropriate range might be calculated in the log transformed scale (see below).

Kirkwood also defines the percent geometric coefficient of variation as

$$\%GCV = 100 \times (GSD - 1)\% \qquad (43)$$

An alternative measure of variability derived from the arithmetic moments of the log-normal distribution in the original scale is

$$\%CV = \sqrt{\exp\left(S_T^2\right) - 1} \times 100\% \qquad (44)$$

Numerically, *%GCV* and *%CV* of the log-normal distribution are close to each other when both are less than 20% (see Tan, 2005). Their use along with *GSD* should be clearly specified when reporting the measure of variability or intervals for log-normal data. Interpretation of these measures are described more fully in *Biological Assay Validation* ⟨1033⟩, *Appendices, Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables.*

From *equation (5)* in *Basic Statistical Principles and Uncertainty*, a 100(1 − α)% two-sided confidence interval on the mean in the log scale is

$$LB(T) = \overline{T} - t_{1-\alpha/2:n-1}\frac{S_T}{\sqrt{n}}$$
$$UB(T) = \overline{T} + t_{1-\alpha/2:n-1}\frac{S_T}{\sqrt{n}} \qquad (45)$$

where n is the sample size and $t_{1-\alpha/2:n-1}$ is the 1 − α/2th quantile of the cumulative Student t distribution having area 1 − α/2 to the left and n − 1 degrees of freedom.

The confidence interval on the geometric mean in the original scale is obtained from the bounds in *equation (45)* as

$$LB(Y) = exp(LB(T))$$
$$UB(Y) = exp(UB(T)). \qquad (46)$$

Transformations other than logarithms may be considered for other types of data. For example, when working with proportions between 0 and 1 (or percentages between 0% and 100%), either the arcsine or logit transformation is useful. The arcsine transformation where Y is represented as a proportion is

$$T = 2 \times \sin^{-1}(\sqrt{Y}) \qquad (47)$$

and the logit transformation is

$$T = \ln\left(\frac{Y}{1-Y}\right) \qquad (48)$$

These transformations are particularly useful when a majority of the data are pushed against the upper bound of 1.0 or the lower bound of 0.0. Count data may be transformed using a square root or a log transformation of the count.

Power transformations, the most common of which are Box-Cox transformations, are also useful re-expressions. These transformations are of the form

$$T = \frac{Y^\lambda - 1}{\lambda} \lambda \neq 0$$
$$= \ln(Y)\ \lambda \neq 0 \qquad (49)$$

where λ is selected to best transform the data set to normality. Information on Box-Cox transformations is provided in Section 6.5.2 of the NIST/SEMATECH e-Handbook of Statistical Methods.

Regardless of the transformation, summary measures and intervals calculated in the transformed scale can be back-transformed to the original scale. In all cases the data should be examined to establish if the transformed measurements exhibit almost uniform variability and are approximately normally distributed.

Assessing Model Adequacy

All models involve assumptions about the processes that generate the data and the data itself. In addition to the assumed functional form, the distribution of the error term in *equations (38)* and *(40)* is of primary importance. Typical assumptions are that the error terms are independent, normally distributed, and have constant variance across the range of responses. When these assumptions are reasonable, statistical models are usually readily interpretable and powerful (i.e., able to measure subtle effects with good precision and discrimination between groups). As attractive as any model might be, it is imperative to check

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for and address violations of the assumptions upon which these models rely. Assessing model adequacy is the process of verifying these assumptions.

There are both graphical and quantitative methods for assessing model adequacy. In many data analysis projects, there are multiple iterations of conversations between researchers and statisticians before selecting a final model. Topics to consider include appropriate transformations of the data, the treatment and design factors of interest, potential candidate models, and assessment of model fit.

Useful tools for assessing model fit include residual plots with both raw and studentized residuals, model-based outlier detection methods, and regression leverage and influence measures. Plots of residuals can be generated in several ways. The most common format is a plot of the residuals on the vertical axis, and the predicted response on the horizontal axis. When the observations on a residual plot increase or decrease in spread along the horizontal axis, this indicates violation of the assumption of constant variance. Any linear or non-linear trend in the residuals suggests the functional form of the model may not be correct, or that an important treatment factor is missing from the model. For example, a curved residual pattern may indicate the need for a quadratic term in the model. Additionally, residuals that fall outside the general cluster of points may be an indication of an outlier. As noted previously, some of these problems may be mitigated with an appropriate transformation.

Normality of the error terms is an especially important assumption if the model is used to predict future behavior. Graphical methods that can be used to monitor this assumption include dot plots, box and whisker plots, and normal probability plots (sometimes called quantile-quantile or QQ plots). These graphical tools are available in many common statistical software packages. Statistical tests of normality are described in Section 1.3.5 of the previously referenced NIST handbook and available in statistical software packages.

Lack of independence typically occurs when data are in some manner "batched" in groups. For example, measurements that are taken from the same plates on an assay are more similar than measurements recorded on other plates. This so-called intragroup correlation can be properly modeled by including a "batch" factor in the model to account for the correlation.

Care should be taken in the assessment of model assumptions. Statistical tests in particular are impacted by the size of the sample. For small samples such tests may be insensitive for detecting departures from the model assumptions. In contrast for large samples, they may detect an assumption violation even though visual assessment suggests the assumptions are reasonable. A combination of scientific understanding of the measurement process generating the data, graphical analyses and statistical tests can be used together to address model adequacy.

Outliers

Occasionally, observed analytical results are very different from expected analytical results. Aberrant observations are properly called outlying results. These outlying results should be documented, interpreted, and managed. Such results may be accurate measurements of the property being measured but are very different from what is expected. Alternatively, due to an error in the analytical system, the results may not be typical, even though the property being measured is typical. A first defense against obtaining an outlying analytical result is application of an appropriate set of system suitability and control rules (see *Appendix 1: Control Charts*).

When an outlying result is obtained, systematic laboratory and process investigations are conducted to determine if an assignable cause can be established to explain the result. Factors to be considered when investigating an outlying result include human error, instrumentation error, calculation error, and product or component deficiency. A thorough investigation should consider the precision and accuracy of the procedure, the USP or in-house Reference Standard and controls, process and analytical trends, and the specification limits. If an assignable cause due to the analytical procedure can be identified, then retesting may be performed on the same sample, if appropriate, or on a new sample. Based on the documented investigation, data may be invalidated and eliminated from subsequent calculations.

"Outlier labeling" is informal recognition of outlying results that should be further investigated with more formal methods. Outlier labeling is most often performed visually with graphical techniques such as residual plots, standardized residual plots, or box and whisker plots. "Outlier identification" is the use of statistical significance tests to confirm that the values are inconsistent with the known or assumed data distribution. The selection of the correct outlier identification technique often depends on the initial recognition of the number and location of the values.

A simple example is presented to demonstrate this process. An analytical procedure requires measurements from three vials of liquid drug product which are used to provide a reportable concentration value (mg/ml) for the lot from which the vials were selected. When measuring the third vial, the analyst noted a slight deviation in the sample preparation which was not discussed in the protocol. The three measurements are reported in *Table 11*. Vial 3 is the vial in question.

Table 11. Concentrations for three vials of drug product

Vial	Concentration (mg/ml)
1	49.9
2	49.8
3	51.8

The residual plot for the mean model described in *equation (38)* is shown in *Figure 4*. Here the residual is the measured value minus the sample mean of the three vials (50.5 mg/ml).

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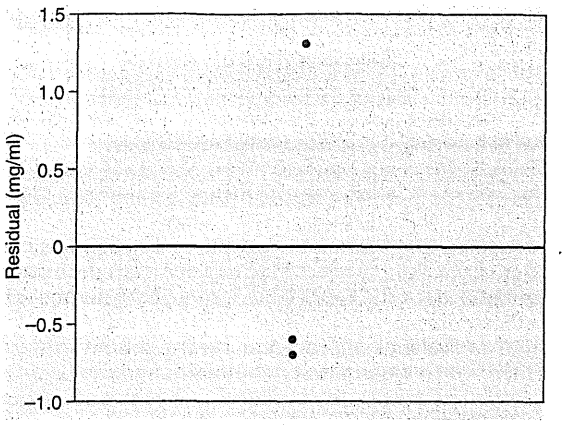

Figure 4. Residual plot of the data.

The residual for vial 3 visually resides far from the other two values and is accordingly *labeled* as an outlier.

One statistical test that can be used to determine if vial 3 can be *identified* as an outlier is due to Dixon (1950, 1951). This test is based on a ratio of differences between the observations. For this particular application where interest is in determining if the maximum value is an outlier, a single test statistic is computed and compared to a critical value based on a normal probability distribution. The minimum value in the data set is 49.8 mg/ml, the middle value is 49.9 mg/ml, and the maximum value is 51.8 mg/ml. The test statistic is defined as

$$\frac{\text{Maximum}-\text{Middle}}{\text{Maximum}-\text{Minimum}} = \frac{51.8-49.9}{51.8-49.8} = 0.95 \qquad (50)$$

The calculated value in *equation (50)* is then compared to a table of values based on the distribution of order statistics for a normal probability distribution. The critical value that must be exceeded to be identified as an outlier with three values using a type 1 error rate of 0.05 and assuming a normal distribution is 0.941. Since the computed value of 0.95 exceeds 0.941, the measurement of vial 3 is identified as an outlier. Actions to be taken will depend on results of further investigations.

As noted, this particular version of the Dixon test requires an assumption of normality which cannot be verified with such a small sample. Rather, one would need to rely on previous measurements made with the procedure on previous process lots to support this argument. In general, the critical value as well as the ratio that one constructs for the Dixon test depends on the number of measurements in the data set and the type 1 error rate. A complete set of critical values for sample sizes less than 30 are available in Böhrer (2008).

As noted previously, the process of identifying a statistical outlier generally requires scientific support for an assignable cause. For the applications performed in an analytical lab, candidate outlier tests are typically univariate. Two questions to consider when selecting a method are

1. Can the distribution be assumed to be normal, or should a test be applied that does not require this particular distributional form?
2. Do we suspect more than one outlier, and which observation(s) have been labeled?

With regard to question 1, outlier tests can be categorized as either parametric (model-based) or non-parametric. The parametric structure selected by such methods is typically the normal distribution. Question 2 considers whether there is one or more labeled outliers, and the relative location (i.e. greater or less than the bulk of the measurements). If more than one outlier is suspected, then sequential approaches may be needed to perform the test.

Useful references on this topic include Barnett and Lewis (1994), Hawkins (1980), ASTM E178, and a literature review by Beckman and Cook (1983).

APPENDIX 3: EQUIVALENCE AND NONINFERIORITY TESTING

General Notices describes the need to produce comparable results to the compendial method. Several options were identified to address this as noted in Hauck et. al. (2009). Among these was performance equivalence. Performance equivalence is used to establish the equivalence of the two procedure means, and noninferiority of the new procedure variability to that of the old procedure, as the basis for demonstrating comparability between two procedures.

The article goes on to describe an approach for demonstrating comparability using statistical hypothesis testing. This appendix describes the general principles of statistical hypothesis testing, as applied to equivalence testing of procedure means and noninferiority testing of procedure variabilities.

In classical statistical hypothesis testing, there are two hypotheses, the null and the alternative. For example, when comparing a new and an old procedure, the null may be that two means are equal and the alternative that they differ. This may be expressed as

$$\begin{aligned} H_0&: \mu_N = \mu_O \\ H_a&: \mu_N \neq \mu_O \end{aligned} \qquad (51)$$

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or equivalently

$$H_0: \mu_N = \mu_O = 0$$
$$H_a: \mu_N - \mu_O \neq 0 \qquad (52)$$

where μ_N and μ_O are the means for the new and old procedures, respectively.

With this classical approach, one rejects the null hypothesis in favor of the alternative if the evidence is sufficient against the null. In such a case we accept the alternative hypothesis that the means are different. Because of this interpretation, this is sometimes called a *difference test*.

A common misinterpretation is to conclude that failure to reject the null hypothesis in a difference test is evidence that the null is true (i.e., the means are equal). Actually, failure to reject the null just means the evidence against the null was not sufficient to claim the means are different. This might occur if the variability is large, or the number of determinations too small to detect a difference in the means.

Thus, when one seeks to demonstrate equivalence of procedure means, it is necessary to place the claim of equivalence in the alternative hypothesis. A statistical test for an alternative hypothesis of equivalence is referred to as an *equivalence test*. It is important to understand that "equivalence" does not mean "equality." Equivalence should be understood as "sufficiently similar" for the use of the new procedure. The definition of "sufficiently similar" is something to be decided a priori based on scientific considerations, and becomes the basis of the alternative hypothesis. Chatfield and Borman (2009) offer some helpful suggestions for this process.

As a specific example, suppose it is decided a priori that to be considered equivalent, the means of two procedures can differ by no more than some positive value, d. This value is commonly called the equivalence margin. The hypotheses for the equivalence test are then

$$H_0: |\mu_N - \mu_O| \geq d$$
$$H_a: |\mu_N - \mu_O| < d \qquad (53)$$

Note the alternative hypothesis is actually two individual one-sided hypotheses:

1. $H_{a1}: \mu_N - \mu_O < d$, and
2. $H_{a2}: \mu_N - \mu_O > -d$.

For this reason, this testing procedure is referred to as two one-sided tests (TOST). As one-sided tests, each can be addressed with a type I error rate of α (typically, but not necessarily, 0.05). The TOST is often conducted by rejecting the null hypothesis in favor of the alternative hypothesis if the $100(1 - 2\alpha)\%$ two-sided confidence interval (typically, but not necessarily 90%) is entirely contained in the range $(-d, +d)$. When the null is rejected, we conclude that the two procedures are equivalent in their means.

Performance equivalence is not restricted to demonstrating equivalence of procedure means. A laboratory might want a new procedure to have equivalent or better variability as the old procedure. This requires a one-sided test because if the new procedure were to have a lesser variability, this would clearly be acceptable. What one needs to ensure is that the new procedure does not result in an important increase in variability. Thus, variability comparisons are conducted as one-sided *noninferiority tests*.

Similar to an equivalence test for means, a noninferiority test for variabilities places the desired relationship between procedure variabilities in the alternative hypothesis. Due to the statistical properties of standard deviations, an appropriate parameter for comparison is the ratio, σ_N/σ_O, where σ_N and σ_O represent the standard deviations of the new and the old procedures, respectively.

Suppose it is determined a priori that for the procedure to be fit for use, the standard deviation of the new procedure can exceed that of the old procedure by no more than a factor $k \geq 1$. The factor k is called the noninferiority margin. The hypotheses associated with the noninferiority test are

$$H_0: \frac{\sigma_N}{\sigma_O} \geq k$$
$$H_a: \frac{\sigma_N}{\sigma_O} < k \qquad (54)$$

Unlike the equivalence test of means, the noninferiority hypothesis is a single hypothesis which can be addressed with a level α (typically, but not necessarily, 0.05). In order to perform the test, the null hypothesis is rejected in favor of the alternative hypothesis if the $100(1 - \alpha)\%$ upper one-sided confidence bound on σ_N/σ_O is less than k. When the null hypothesis is rejected, it is concluded that the variability of the new procedure is noninferior to that of the old procedure.

Hauck, et.al. offers other options to address the standard of "equivalent or better":

1. minimum performance requirements for acceptable procedures,
2. results equivalence, and
3. decision equivalence.

The option of minimum performance requirements has evolved into the concept of the analytical target profile (ATP) which has been introduced in *Pharmacopeial Forum* (Barnett et al. 2016). Results equivalence is addressed using the intra-class correlation coefficient or the concordance correlation coefficient. A tolerance interval approach using total variability is likewise used to address results. Decision equivalence relates to dichotomous outcomes such as pass/fail, and can be addressed through the kappa coefficient or receiver operating characteristic curves. Using these options (as with performance equivalence), care

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must be taken to properly formulate the statistical hypotheses and to address the comparison through meaningful acceptance criteria.

While this appendix has highlighted approaches for establishing procedure comparability, these apply to other scenarios involving comparisons of two groups; e.g., procedure transfer or standard qualification. Placement of the claim one desires to support into the alternative hypothesis results in an appropriate statistical conclusion.

Although the benefits of equivalence testing are apparent, in some situations one may not be able to collect a sufficient sample size to provide the necessary power to establish equivalence. In such a situation, use of the difference test may be the only option. However, one is reminded that failure to reject the null hypothesis of equality is not evidence that the procedure means are equal. A confidence interval should nonetheless be reported to communicate the difference of means between the two procedures.

APPENDIX 4: THE PRINCIPLE OF UNCERTAINTY

While this chapter has concentrated on statistical studies which are performed using measurement data, the principles and practices are identical to those in the field of metrology. These are unified by a common understanding of the concept of uncertainty. This appendix introduces concepts related to the metrological principle of measurement uncertainty and unify these with the practices described for the scientific method.

The understanding of study uncertainty is not new to the pharmaceutical industry and has been employed more broadly throughout industries that make decisions from studies using measurements. The study of measurement uncertainty falls formally into the field of metrology. A measurement process like a study is designed to reduce uncertainty in order to make a more informed decision. No measurement or study result can provide exact knowledge. Proper interpretation and treatment of analytical data requires an understanding of the inherent sources of uncertainty in measurement outcomes and their impact on the information they provide. Recognition of the principles of uncertainty facilitates this understanding, as described by the Joint Committee for Guides in Metrology in the *Guide to the Expression of Uncertainty in Measurement* (GUM).

Results from all studies, including quality control testing are uncertain. Uncertainty arises from sources of variability inherent in the measurement process, as well as from statistical sampling and study factors. The principles from the field of metrology are consistent with the statistical principles described in this chapter and provide further insight into the quantification of uncertainty from studies supported by measurements.

At the core of these principles is an understanding of risk. More specifically, this understanding considers the risks of making incorrect decisions based on studies utilizing measurements. The consequences of these risks can be minor or significant, and thus should be factored into considerations related to the design of a measurement system, the design of studies using the measurement system, and the interpretation of study results. The concepts of Target Measurement Uncertainty (TMU) and the study objective can be unified as a basis for managing the risks associated with making decisions from studies. In fact, TMU is a special case of a study hypothesis which drives the design of all studies using analytical measurements.

To increase knowledge, two of the fundamental forces of metrological and statistical thinking are the desire to minimize the uncertainty in the measured value (an indication of the quantity being measured) and to ensure all sources of uncertainty have been evaluated and mitigated. In metrology the quantity intended to be measured is termed the measurand. This is called a population parameter in the broader sense of a study. Measurement or parameter uncertainty quantifies one's doubt about the true value that remains after making a measurement or estimating a parameter.

While the metrological concept of measurement uncertainty applies exclusively to a reportable value, this can be aligned with the concept of study uncertainty by viewing the quality control process as a study of a commercial lot. Employing the steps of the scientific method, the study of the commercial lot has an objective which can be formulated as a hypothesis test

$$H_0: \mu \leq \text{LSL or } \mu \geq \text{USL}$$
$$H_a: \text{LSL} < \mu < \text{USL} \qquad (55)$$

where μ is the commercial lot mean and LSL and USL are the lower and upper specification limits respectively. The study can be designed using blocking and replication to satisfy the TMU, which should be such as to minimize the risks associated with the object of the testing (i.e., to support the alternative hypothesis, H_a). As part of study conduct, sampling and randomization can be utilized to mitigate the risks due to the introduction of bias. Finally, and perhaps most importantly, the data should be analyzed and reported with acknowledgement of the uncertainty in the reportable value.

Metrological Principles Specific to Measurement Uncertainty

The reliability of study results are only as good as the fitness for use of the measurement process used to generate data for the study. The metrological concept of measurement uncertainty helps to ensure fitness for use. This and other principles are worth noting as a fundamental way to view a measurement process.

Figure 5 represents several potential sources of random variation in a measurement process, which result in the combined standard uncertainty (the estimated standard deviation of the measurement). An example of inherent random variation is when the same chromatogram is given to several different analysts for peak integration. Slightly different values will be obtained which might also be affected by a laboratory's choice of software. In addition, the definition of the measurand can never be complete. This is known as definitional uncertainty or uncertainty of knowledge. Ideally the measurand is defined sufficiently so that the definitional uncertainty is relatively small when compared to the combined standard uncertainty. An example of lack of knowledge is when a component of the measurement process has associated uncertainty. For example, one might purchase a pH standard solution that is certified as pH = 7.00 ± 0.02 where the 0.02 is the expanded uncertainty in the assigned

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value of the standard solution. Expanded uncertainty is a measure of uncertainty that defines an interval about the measurement result y within which the value of the measurand Y can be confidently asserted to lie.

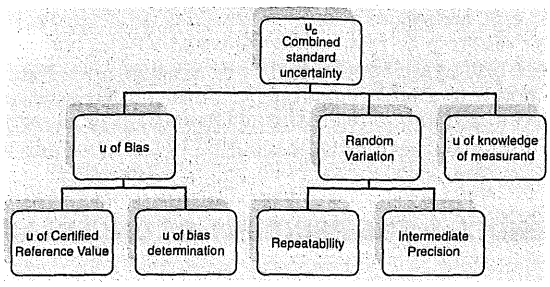

Figure 5. Various components of uncertainty (u) comprise the combined standard uncertainty. The figure is not comprehensive and is meant to illustrate the major uncertainty components.

GUM notes the evaluation of measurement uncertainty is neither a routine task nor a purely mathematical one. Judgment is essential in choosing which uncertainty components (i.e., potential causes of measurement uncertainty) to consider in procedure development, qualification, and measurement uncertainty evaluation. For example, when preparing a 1 mg/L solution, the process by which it is prepared can influence the final concentration. The analyst knows it would not be wise to weigh 1 mg of the substance followed by dilution to 1 L. Instead, recognizing the limits of typical analytical balances, a more precise result would be achieved by weighing 100 mg of substance and then serially diluting to the required concentration.

Measurement uncertainty arises from many sources including differences in instruments, mathematical algorithms, and analysts. A tutorial sampling of typical uncertainty components is provided in *Table 12*.

Table 12. A tutorial list of uncertainty components in analytical laboratory practice

Variability due to analytical procedure design	Effect of the sample amount or volume Between-unit variation of measurand Purity of the primary standard Effect of the sample storage conditions Failure to recognize ruggedness factors
Variability due to measurement process	Carry-over effects in the auto sampler Effect of static electricity on weighings Incomplete recovery of the analyte The effect of the sample matrix on calibration slope Effect of the sample temperature on the volume Effect of the blank correction
Variability due to analysts	Effect of the manual peak integration
Variability due to algorithms	Linear calibration forced through zero Line fitting using weighted or unweighted algorithms
Variability due to sample	Effect of taking a sub-sample from a laboratory sample

A detailed discussion of measurement uncertainty in the pharmaceutical industry that expands upon the metrological principles introduced here and provides detailed definitions is provided in Weitzel et al. (2018). In addition, a worked example for a drug substance is provided in Weitzel et al. (2017).

APPENDIX 5: BAYESIAN INFERENCE

When describing statistical intervals in *Section 4, Basic Statistical Principle and Uncertainty*, it was noted that one can utilize a Bayesian approach to derive an interval which contains, with probability $100 \times (1 - \alpha)\%$ the true value of the population mean. This is important because it returns a statement that the laboratory frequently wishes to make. This section will describe Bayesian inference and contrast it with frequentist inference which is more commonly understood throughout the pharmaceutical industry. Frequentist theory bases inferences on probability statements about statistics, while Bayesian inference is based on probability statements about population parameters. Population parameters are the unknowns that appear in statistical models (e.g., means, variances, difference of means) and statistics are summary measures or estimates based on data (eg., parameter estimates). Frequentist inference regards parameter values as fixed and unknowable whereas Bayesian inference models their uncertainty using probability distributions. For instance the statement "there is a 95% probability that the difference in population means is between –0.1 to 0.1" is meaningless from a frequentist viewpoint, but reasonable from a Bayesian perspective. The Bayesian formulation offers a way for scientists needing to make risk based decisions. Bayesian inference can also incorporate prior information about statistical parameters together with the sample data to update what is known about a parameter. The ability to incorporate justified prior information potentially leads to better decisions when a study size is small, or when a factor is not adequately represented in the study design.

The purpose of this appendix is to provide a basic introduction to Bayesian inference applied to statistical studies and to analytical measurements. Gelman et al. (2013) provides a source for more information.

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Parameter Uncertainty versus Sampling Variability

Parameters are unknown hypothetical or population quantities, such as the mean or standard deviation of a population, or the difference in means between procedures. While unknown a parameter can be estimated. The estimation of a parameter and the inherent uncertainty of that estimation is the basis of Bayesian thinking.

Statistics are observed quantities or summaries of observed quantities in a sample taken from a population or process of interest. Examples of statistics include an analytical result (a measurement), a sample mean, a sample standard deviation, a difference in observed means between procedures, or their estimated confidence bounds. On repeated sampling of the population, the observed values of *statistics* will differ because of *sampling variability*.

Frequentist statistical methodology considers parameters to be fixed values that do no change. It employs probability theory to model the *sampling variability* of *statistics* randomly obtained from the population. These sampling distributions are then used to make inferences about the fixed value of the *parameter*. A common frequentist methodology is the calculation of a confidence interval. The process of computing a 95% confidence interval ensures that the realized interval will contain (or cover) the unknown parameter 95% of the time on repeated use.

The 95% refers to the reliability of the methodology (i.e., its coverage), and not the probability that the parameter falls within the interval.

For example, suppose a computed 95% confidence interval on a mean is from 980 to 990 mg/g. It is not correct to state there is a 95% probability that the population mean is between 980 and 990 mg/g. To associate a probability with a fixed interval such as 980 to 990 mg/g, one must assume uncertainty is associated with the underlying parameter (i.e., it is not a fixed quantity). Rather, the 95% description of the confidence interval means that the interval will correctly contain the true parameter value in 95% of repeated sampling applications from the population. The 95% refers to the success rate of the sampling process and not the parameter (which is assumed fixed).

Bayesian statistical methodology considers a parameter value to be uncertain (not fixed), and models its likely levels using a probability distribution. It extends frequentist statistical methodology, using probability theory to model both the sampling variability of statistics and the decision maker's uncertainty associated with parameters. Bayesian models are sometimes called "complete" probability models because they quantify the uncertainty associated with the parameters of interest, given the assumed sampling variability of the observed statistics, any relevant prior information, and the observed data. For instance, it *is* correct to say that a given Bayesian 95% credible interval (Bayesian analogue of the confidence interval) contains the value of a specific parameter of interest with 95% probability (conditional on the observed data and other modeling assumptions). The same principles apply to the Bayesian analogues of frequentist tolerance and prediction intervals. Unlike frequentist interval methodology in which the probability level must be fixed in advance (e.g., 95%) and the resulting interval is random, Bayesian methodology offers the opportunity to fix the interval in advance, and estimate the probability that the parameter value lies within that interval. Such an application is extremely useful for determining the probability that an analytical procedure will provide a signal outside a given range.

Prior and Posterior Distributions

Both frequentist and Bayesian methodologies express models using probability distributions. Both use the same model for sampling variability known as the likelihood. The particular likelihood model choice is based on prior knowledge concerning statistical variability.

Bayesian inference also requires a probability model for parameter uncertainty, prior to observing the data, called the *prior distribution*. As with the likelihood, the prior distribution is a choice based on prior data, reliable knowledge, or common sense (e.g., the values of many parameters, such as a standard deviation, must be positive). Bayesian methodology requires care to assure that the chosen prior distributions are scientifically justified and do not unduly influence the inference. Use of appropriately justified knowledge of a prior distribution can potentially reduce sample size requirements for decision making. However, when there is little available theory, historical data, or expert knowledge available, prior distributions can be constructed that give minimal preference to any particular parameter value, and thus have minimal impact on the inference. Such prior distributions are often referred to as "non-informative". When non-informative prior distributions are employed, inferences typically agree with the frequentist counterparts since both are solely dependent on the likelihood.

Bayesian methodology combines likelihood and prior distributional models with observed data to produce an updated distributional model for parameter uncertainty called the *posterior distribution*. The posterior distribution provides the probability that the population parameter value lies within any interval of interest. Such intervals are called credible intervals. When certain classes of non-informative prior distributions (e.g., a Jeffrey prior used with a normal likelihood) are employed, a Bayesian credible interval can be calculated from the posterior distribution, and may sometimes be numerically equal to the corresponding traditional confidence interval. However, as previously noted, the interpretations of these intervals are different. The probability associated with the credible interval quantifies uncertainty in an estimated parameter value conditional on observed data, while the probability associated with the confidence interval quantifies the probability of coverage of the estimated parameter on repeated estimation over many data sets.

From the Bayesian perspective, all knowledge about the parameter of interest is based on the posterior distribution. The posterior distribution from a previous study can inform the prior distribution for a subsequent study. Updating the prior distribution in this manner as new data become available, provides a paradigm for knowledge building, and thus a statistical basis for applying *prior knowledge* during pharmaceutical development (see ICH Q8(R2), *Pharmaceutical Development*).

The posterior distribution of parameters may also be re-combined with the likelihood to obtain a *posterior predictive distribution* of future observed data or statistics. As with the posterior distribution, the Bayesian perspective bases all knowledge about future values on this posterior predictive distribution, which can be used to construct Bayesian analogues of frequentist tolerance and prediction intervals. Unlike the frequentist analogues, the Bayesian intervals do not require a pre-specified fixed

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probability level. A posterior predictive distribution can be used, for example, in estimating the probability of occurrence of future out-of-specification results.

An Illustrative Example

Consider an analytical procedure for strength of drug product. The output of the procedure is a reportable value (mg/g) that estimates the mean strength, μ, for the tested lot of drug product. For the lot to be considered safe and effective, μ must be between 980 and 1020 mg/g. The observed reportable result, Y, is 1010 mg/g.

A typical rule used for disposition is to release the lot if $980 \leq Y \leq 1020$. However, this rule is based on an observed reportable result that includes measurement error from the analytical procedure. What we really want to know is whether μ falls within the specification limits. This question can be informed using a Bayesian rule that releases the lot if the posterior probability that $980 \leq \mu \leq 1020$ is above some lower limit (e.g., 0.95). That is, we release the lot if the probability that the true value is within specifications is at least 0.95. Such a rule might be called a minimum posterior probability (MPP) rule. The MPP rule provides a probability based metric for acceptance of the lot under test.

The estimation of this posterior probability requires definitions of the likelihood model and its parameters, the prior distributions of these parameters, and the data. For this illustration, the following are assumed:

- *Likelihood*: reportable results follow a normal distribution with two unknown parameters: the population mean (μ) and intermediate precision standard deviation (σ). [NOTE—It is assumed the lot is homogeneous.]
  - **Prior distributions:Prior distribution of μ**—There is no prior information on the strength of this lot. To represent this lack of knowledge assume a wide uniform distribution over the analytical range. The uniform distribution gives equal probability to any range of a given length regardless of location.
  - **Prior distribution of σ**—Data collected during validation resulted in an estimated intermediate precision variance (σ_0^2) of 25 based on a sample of 10 independent reportable values. Based on this information, assume that σ^2 follows a scaled-inverse-chi-squared distribution (a common prior distributional choice for variances) having $df_0 = 10 - 1 = 9$ prior degrees of freedom and a prior scale parameter of $\sigma_0 = \sqrt{25} = 5$ mg/g.
- *Data*: a reportable value, $Y = 1010$ mg/g

Given the above information, the Bayes rule leads to a Student-t posterior distribution for $(\mu - Y)/\sigma_0$ with $df_0 = 9$ degrees of freedom. The integration of this posterior distribution over the fixed range for μ of 980 to 1020 mg/g can be conveniently obtained using commonly available spreadsheet functions. For example, in Excel this is computed using the formulas, =T.DIST((1020–1010)/5,9,TRUE) – T.DIST((980–1010)/5,9,TRUE). The resulting posterior probability that $980 \leq \mu \leq 1020$ for the tested lot is 0.96. That is, there is a $100 \times 0.96 = 96\%$ chance that the true mean of the lot falls within the specification limits. Because $0.96 > 0.95$, the lot would be accepted based upon the MPP rule. The estimated posterior probability of 0.96 serves as a quantitative risk-based measure of the quality of the lot.

In this example, the parameter of interest is μ, a measurand quantity value. An analogous approach is used for Bayesian inference of other model parameters, such as the estimation of the difference in population means for two procedures, the underlying slope and intercept in a simple linear regression model, or performing tests of statistical equivalence.

In more complex situations (e.g., for complex, non-normal, or non-linear models), Bayesian inference utilizes a form of computer simulation referred to as Markov-Chain Monte-Carlo (MCMC) simulation which is conducted using specialized software. MCMC technology requires care to assure that the MCMC iterations converge properly to the population posterior distribution.

A Comparison of Frequentist and Bayesian Methods

Both frequentist and Bayesian approaches to inference are useful. Frequentist approaches are widely available, straightforward, and offer the reliability of known coverage probability. Bayesian approaches can be used to quantify the uncertainty in parameters of interest which can support quantitative risk based decision making. While often more technically challenging to apply, Bayesian MCMC methodology can often be applied to problems that are intractable by frequentist approaches. When informative prior distributions can be justified, Bayesian methods may require smaller samples sizes for decision making than frequentist statistical methods. *Table 13* provides a comparison of some characteristics from both frequentist and Bayesian perspectives.

Table 13. Characteristic differences between frequentist and Bayesian inference

Characteristic	Frequentist Inference	Bayesian Inference
Statistics	Sampling variability modeled probabilistically	
Parameters	Treated as fixed and unknown	Uncertainty modeled probabilistically
Coverage probability	Known from theory (usually)	Must be determined via computer simulation (usually)
Prior information	Introduced via sampling variability model	Introduced via sampling variability model and prior distributions of parameters
Types of estimates	Point and interval	Posterior distribution (from which point and interval estimates can be derived)
Observed data	Treated as one realization of a hypothetical series of repeated samples	Treated as fixed values on which inference is based

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Table 13. Characteristic differences between frequentist and Bayesian inference *(continued)*

Characteristic	Frequentist Inference	Bayesian Inference
Parametric Inference	Fixed probabilities based on repeated sampling coverage probability	Parameter values quantified probabilistically from posterior distribution
Impact of statistical design	May impact repeated sampling coverage probability	Less critical
Multiple comparisons	May impact repeated sampling coverage probability	Less critical
Risk assessment	Indirect risk assessment	Estimated probabilities appropriate for quantifying risk
Prior knowledge of parameter values	Excluded from the inference	A prior distribution for model parameters is required.
Continuous knowledge building	Informal assessment of historical studies	Posterior distribution from historical study informs prior distribution for subsequent study
Prediction of future observed values	Indirect inference based on tolerance or prediction intervals	Direct probabilistic inference based on the posterior predictive distribution
Software	Widely available routines	Specialized expertise required

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⟨1024⟩ BOVINE SERUM

INTRODUCTION

Bovine serum is the liquid fraction of clotted blood, obtained from an ox (*Bos taurus,* among others), that has been depleted of cells, fibrin, and clotting factors. Since the advent of modern cell culture, manufacturers of biological products have used bovine serum extensively as a cell culture growth supplement. Its rich nutritional composition of proteins, growth factors, hormones, amino acids, vitamins, sugars, lipids, trace elements, and other components supports a broad range of cell culture applications in research and commercial manufacture of vaccines, natural source and recombinant biologics (hereafter biologics), engineered tissues, and other emerging cell-based therapeutic products intended for human or veterinary use. The predominant type of serum used in research applications is Fetal Bovine Serum (FBS). Calf serum (from newborn and older animals) is used much less frequently, but because of its lower cost it may be widely used in commercial manufacturing.

As is the case with other animal-derived products, bovine serum carries a potential risk of introducing extraneous agents into cell culture. Serum manufacturers and regulators must adopt rigorous sourcing and testing procedures and strict processing and production guidelines to ensure the quality of bovine serum.

The objective of increasing the quality and safety of biologics produced with bovine serum, coupled with attempts to mitigate regulatory burden, have caused developers to investigate alternatives to serum supplementation, resulting in application-specific serum-free medium formulations. Although it is recognized that bovine serum should be avoided when there is an option to use serum-free medium, there are cases where this is technically impossible or impractical.

This chapter describes issues related to sourcing, production, and characterization of bovine serum to ensure its safe use. A list of relevant regulatory and guidance documents is presented in *Appendix 1*. Serum manufacturers and serum end users (manufacturers of biological products) should consider and apply as needed the controls and procedures outlined in this chapter to ensure the safe use of bovine serum components in research and pharmaceutical manufacturing.

Types of Bovine Serum

- FBS is obtained from the fetuses of healthy, prepartum bovine dams that had been deemed fit for human consumption through ante- and postmortem inspection by licensed veterinarians. It is collected in government-inspected and -registered slaughterhouses.
- Newborn calf serum (also known as newborn bovine serum) is obtained in government-inspected and -registered slaughterhouses from animals aged less than 20 days.
- Calf serum is obtained in government-inspected and -registered slaughterhouses from animals aged between 20 days and 12 months.
- Donor bovine serum (also known as donor calf serum) is obtained by the repeated bleeding of donor animals from controlled government-inspected and -registered donor herds. The animals are 12–36 months old.
- Adult bovine serum is obtained in government inspected and -registered slaughterhouses from cattle older than 12 months that are declared fit for human consumption.

BOVINE SERUM: HISTORY AND TYPES OF USE

History of Bovine Serum Use

Animal serum and other complex biological materials have been employed in the cultivation of mammalian cells for approximately 100 years. Several factors led to the wide adoption of bovine serum as a standard tissue culture supplement. In comparison to serum from other animal species (horse, goat), bovine serum is easily sourced, and thereby more affordable. Many investigators choose to use fetal serum in their experimental systems because of concerns associated with antibodies present in newborn and adult serum that could cross-react with cells in culture and cause cell lysis through complement-mediated pathways. To eliminate that concern, heat was introduced to inactivate complement that was potentially

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present in the serum. Studies of FBS undertaken in the 1950s on the cultivation of low-density human cells to elucidate mechanisms of cell growth found that (1) the albumin component may serve as a carrier of essential small molecules; (2) fetuin, a glycoprotein present at high levels in the alpha globulin fraction, facilitates cell attachment and stretching; and (3) fetuin markedly inhibits trypsin, and this antiproteolytic activity may play a role in the ability of fetuin to stimulate cell growth.

In the 1960s and 1970s, serum supplementation of tissue culture media became the norm, thus facilitating biomedical research as well as the first large-scale vaccine manufacturing processes. Serum supplementation reduced the requirement for optimizing medium formulations for different cell types. FBS was shown to provide a variety of polypeptide growth factors. Albumin promoted cell growth presumably because of its abilities to function as a carrier protein for small molecules or lipids, to bind metal ions, to serve as a pH buffer, and to protect cells against shear. Similar functions were found for other serum components such as transferrin, hormones, and other serum-derived attachment factors such as fibronectin, vitronectin, and laminin.

Uses of Bovine Serum

Serum is a complex mixture of macromolecules that is required for cell growth and virus production, and its use as a raw material presents a number of challenges. These include its batch-to-batch composition and the risk of contamination by adventitious agents. The development of serum-free media has replaced serum in some new biotechnology manufacturing applications, but many cell lines used in manufacturing have not been adapted to these serum-free media. Regulatory constraints and scientific challenges generally make it impractical to alter existing manufacturing processes in which serum is used as a raw material.

FBS sometimes is required in cell and tissue bioprocessing, which often involves the cultivation of cells from tissue explants and biopsies. Some bioprocesses may also require the maintenance of specific cellular characteristics during cultivation. FBS often appears to facilitate such procedures and may affect the biological behavior of fastidious cell types. FBS has been shown to affect the transcription of developmentally important genes, apoptosis, and apoptosis-related gene expression, and to provide neuroprotective and antioxidative factors, all of which may be beneficial to the survival and development of cells in culture. Therefore, FBS will continue to play an important role as a cell culture supplement for production of cell- and tissue-based therapies.

In most viral vaccine manufacturing processes the media used for cell culture expansion and virus infection/production are supplemented with different types of serum at different concentrations. In these processes, bovine serum helps generate a mass of cells in an optimal physiological state for efficient viral replication.

BOVINE SERUM HARVESTING AND PRODUCTION

Blood Collection

For all types of bovine sera, blood should be collected in government-inspected and -registered premises (slaughterhouses, abattoirs, and donor farms). Blood should be collected by trained operators following the written procedures approved by the serum manufacturer and using either single-use disposable collection devices or reusable collection equipment for which cleaning procedures have been validated.

DONOR BOVINE SERUM

For each lot of serum from donor animals, serum manufacturers should ensure traceability to the donor herd of origin via production records and animal health and origin certificates. Donor animals are subjected to regular veterinary inspections and are bled multiple times following established procedures. Animals introduced into the herd should be traceable by source, breeding, and rearing history. Collectors should introduce new animals into the herd following specified and approved procedures that include prepurchase animal inspection and testing, proper transportation, a quarantine period, veterinary examination and testing during the quarantine period, and animal release criteria from quarantine to serum production. The collectors should not vaccinate donor animals for bovine viral diarrhea (BVD). Collectors should test animals for any agent and antibody from which the herd is claimed to be free.

NEWBORN CALF SERUM, CALF SERUM, AND ADULT BOVINE SERUM

Certificates of animal health and origin and/or serum production records should ensure that serum manufacturers can trace bovine serum derived from slaughtered animals back to the abattoir. Serum manufacturers should require abattoirs to maintain documentation of the origin of animals for slaughter. Blood should be collected from animals that have been slaughtered, for human consumption, in abattoirs inspected by the competent authority of the country of origin. Inspectors should routinely inspect animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. Blood collection procedures must be in place to prevent cross-contamination with other tissues and body fluids and the surrounding environment. The standard procedure of slaughter consists of an approved method of animal stunning followed by exsanguination.

FETAL BOVINE SERUM

FBS product specifications and test procedures are presented in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩. Serum manufacturers should collect fetal bovine blood from bovine fetuses whose dams have been slaughtered. The dams must have been deemed fit for human consumption and must have been slaughtered in

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abattoirs that were inspected by the competent authority of the country of origin. Inspectors should examine all animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. The uterus is removed and transported to a dedicated space for fetal bovine blood harvest, where blood collection personnel evaluate the fetus for signs of fetal death, including bloating, skin discoloration, odor, deformation, and hair sloughing. Collectors also should check the amniotic fluid for color, quantity, and clarity. Serum manufacturers should collect blood from acceptable fetuses by cardiac puncture into a closed collection system under conditions designed to minimize microbial contamination. Manufacturers should have in place procedures that will prevent cross-contamination with other fetal tissues and bodily fluids and the surrounding environment.

Serum Harvesting and Processing

Trained personnel should perform serum separation (harvesting) and further processing activities following written and approved procedures. Serum is first separated and pooled, followed by filtration and filling into clean and disinfected containers. If the serum is subjected to one or more virus inactivation treatments in the production process, serum manufacturers should validate the virus inactivation processes against a range of relevant viruses. It is recommended that bovine viral diarrhea virus (BVDV) be included in any virus validation study because it is ubiquitous.

SERUM SEPARATION AND HARVESTING

Bovine blood should be processed and serum separated (harvested) in such a way as to minimize bacterial and mycoplasmal contamination, which in turn minimizes endotoxin levels in serum product. Gentle, quick blood processing helps to minimize hemolysis, further enhancing the quality of the serum product. After collection, blood is first allowed to clot for a specified period of time and under controlled conditions, then centrifuged in a refrigerated centrifuge. Serum is then removed from the clot, typically by centrifugation; pooled and mixed in a pooling vessel; transferred to labeled containers; and frozen, unless it is filter-sterilized immediately. Serum manufacturers should describe each process step and carry out serum processing activities, including sample collection and in-process quality control testing, following the manufacturer's approved procedures.

POOLING BEFORE FILTRATION

Because limited amounts of blood can be collected from individual animals, serum manufacturers pool the raw serum from many animals in order to create commercial-sized lots. Serum is pooled, after raw serum thawing and before filtration, in a pooling vessel and mixed at a controlled mixing rate and temperature. Pools or lots of donor bovine serum may consist of many separate collections from individual members of the herd. Lots of FBS may consist of pooled serum from thousands of animals. Serum manufacturers should describe each prefiltration pooling process step and should carry out serum thawing, prefiltration pooling, and mixing activities following the manufacturer's approved procedures.

FILTRATION

Pooled serum is mixed and aseptically passed through filters of pore size 0.2 µm or smaller, depending on the intended application. Filtration processes should be validated. Triple filtration using filters of pore size 0.1 µm has been shown to result in a high degree of mycoplasma removal. Although filtration may remove some large viruses and viral aggregates from the serum, generally viruses cannot be completely eliminated in this manner. Furthermore, the filters are not known to eliminate the causative agent of bovine spongiform encephalopathy (BSE). Following filtration, serum manufacturers fill filtered serum into sterile containers by aseptic processing in a suitably controlled environment. Serum manufacturers should describe each filtration process step and should perform serum filtration, filling, and sample collection activities following the manufacturer's approved procedures.

IRRADIATION

Serum treatment by gamma irradiation is very common and one of the most effective methods of virus inactivation. The most frequently used minimum dose is 25 kilograys (kGy). Some countries specify higher dose requirements (>30 kGy) for imported serum. Gamma irradiation may inactivate viruses, mycoplasma, and bacteria, but serum end users should ensure that the gamma irradiation process does not negatively affect their specific applications. Irradiation may have adverse effects on serum quality, and these adverse effects tend to increase with higher doses.

Validation of gamma irradiation has two aspects: (1) dose delivery in a defined load configuration and (2) inactivation capacity. Critical irradiation process parameters include product (serum) temperature, packaging size and configuration, dosimeter distribution, and defined minimum/maximum dose received. Dosimeters should be used to monitor the established high-dose and low-dose positions in each irradiation run. If the serum manufacturer makes inactivation claims, these should be supported by the manufacturer's own well-designed viral inactivation studies.

ULTRAVIOLET (UV) TREATMENT

Serum manufacturers may use UV treatment to inactivate viruses, mycoplasma, and bacteria, but manufacturers must validate the process to demonstrate its efficacy. In addition, manufacturers must be aware that UV treatment may have an adverse effect on serum quality and accordingly should consider the effects of UV treatment for each application, as should serum end users.

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HEAT INACTIVATION

Heat inactivation involves elevating the temperature of the serum to >56° for at least 30 minutes to inactivate complement. Heat inactivation may also inactivate viruses, mycoplasma, and bacteria; but it may have an adverse affect on serum quality, and manufacturers must validate the procedure's suitability for specific applications. Heat inactivation provides significantly less assurance of virus inactivation than does irradiation.

VIRAL CLEARANCE STUDIES

Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin ⟨1050⟩ and other regulatory documents give guidance about conducting viral clearance studies that help validate removal/inactivation processes. Serum manufacturers should also perform formal spiking studies with relevant and representative (model) viruses, and should test and compare inactivated spiked serum samples, negative controls, and positive controls.

CHARCOAL STRIPPING

Some serum manufacturers use charcoal/dextran treatment to reduce the levels of hormones in serum.

DIALYSIS

Some manufacturers use dialysis or diafiltration to remove low molecular weight components from serum.

CLEANING AND STERILITY OF EQUIPMENT

Stainless steel systems and tubing used in the manufacture of bovine serum must be cleaned and sterilized to prevent cross-contamination and growth of adventitious agents. Serum manufacturers must validate their cleaning processes for removing and inactivating agents of concern. Thereafter, manufacturers should implement process controls that routinely verify cleaning cycles. Steam sterilization-in-place is a common and effective sterilization technique. Serum manufacturers that use this technology must validate steam cycles to demonstrate their uniformity and ability to destroy heat-resistant bacterial spores. Alternatively, manufacturers can use sterile disposable systems that do not require cleaning validation.

Quality Control

TRACEABILITY

Abattoir Collection: Materials collected in the U.S. should originate from U.S. Department of Agriculture (USDA)-registered facilities. Serum manufacturers should maintain documentation that traces a given serum sub-lot to the abattoir where it was collected. Slaughterhouses maintain records of animal source. General industry practice is to keep this information as part of the Device Master Record. General record-keeping requirements at USDA-licensed abattoir facilities are outlined in 9 Code of Federal Regulations (CFR) 320.

Materials collected from countries approved by the USDA for importation of bovine products into the U.S. should meet the requirements of the competent authority of the country of origin. In addition, serum manufacturers should keep USDA-required safety testing records of imported materials (if applicable) as part of their Device History Record.

Serum manufacturers should consult 9 CFR 309 and 9 CFR 310 about requirements for inspection of animals for various diseases pre- and post-slaughter. These requirements are recommended for materials collected outside the U.S.

Donor Herd Collection: Serum manufacturers should maintain traceability to the donor animal farm where blood was collected from donor animals. In most cases, manufacturers individually identify farm animals and keep records for bleed and processing dates, making it possible to trace blood collection to an individual animal. A licensed veterinarian or a designee under the guidance of a veterinarian should inspect animals regularly and should certify that the animals are free of disease and fit for human consumption, consistent with 9 CFR 309.

PRODUCT STORAGE AND STABILITY

Serum should be stored in the frozen state at −10° or below. Serum is frozen as quickly as possible to preserve product quality and is stored under controlled storage conditions. Serum manufacturers should establish serum product stability in support of a proposed expiration date. Typical expiration dating for bovine serum is 5 years from the date of filtration and filling. Use of any type of bovine serum beyond the stated expiration date depends on the application, and the serum user must establish the product's continued suitability for use.

Labeling

Finished product labels must contain the following information: product description, lot number, storage conditions, name and address of manufacturer, and a statement indicating the intended use. Materials intended for research purposes are exempt from labeling regulations (21 CFR 801). Typically, serum manufacturers supply a lot-specific Certificate of Analysis (COA) that is classified as part of the product's labeling. See COA requirements in the following section.

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Certification/Documentation

CERTIFICATE OF ANALYSIS

The COA should provide information about a specific lot of serum, including tests performed and test results (according to the serum manufacturer's specifications for release), as well as critical labeling identifiers such as lot number, catalog number, description of type of bovine serum, country of origin, and either or both dates of manufacture and expiration. This document is distinct from the certificate of health issued by the competent authority of the country of origin.

CERTIFICATE OF ORIGIN AND CERTIFICATION OF ANIMAL STATE OF HEALTH

The Certificate of Origin establishes the country in which the bovine blood was collected and veterinary certification of the health of the animals pre- and postcollection (9 CFR 327.4).

IMPORT/EXPORT DOCUMENTS

Import/export documents contain formal certification of animal disease status of the country of origin and negotiated/agreed certification statements. These vary from country to country. Each country defines import/export requirements in order to control introduction of exotic animal diseases and their economic impact as well as product safety assessments (risk vs. research, diagnostic, and/or therapeutic benefits).

PRODUCTION REPORTS

Production reports typically are batch records that document the raw materials in identifiable and traceable ways, production methods (centrifugation or filtration) used in manufacturing, equipment and facility cleaning, quality control testing, and personnel performing required activities. Raw material with Certificates of Origin or serum production records facilitates traceability to the source of the blood that was used to create the serum. When serum is used as a raw material for further manufacturing, process documentation also helps demonstrate controlled manufacture of the bovine serum.

BSE RISK ASSESSMENT

Despite the low risk potential of transmissible spongiform encephalopathies (TSEs) in bovine serum, various U.S. and international regulatory agencies have developed guidance to help manage and further reduce the potential risks of transmission. In the absence of appropriate test methods of detecting the infectious agent in fluids such as blood, the consensus recommendation from various regulatory agencies is to adopt good risk assessment strategies. This section of the chapter provides some background information on the disease and current methods of detection; it also highlights risk assessment and risk reduction strategies to potentially prevent transmission of the disease through the use of serum in the manufacturing of medicinal products.

Description of the Disease

TSEs are transmissible animal and human diseases that are characterized by degeneration of the brain, associated with severe neurological signs and symptoms. Since the outbreaks of TSE in cattle, termed BSE, which were transmitted to other species, public health officials have been concerned about the risk of TSE infection, including the possibility of TSE transmission by the use of therapeutic products manufactured using bovine serum. In cattle infected with BSE, lower titers have been found in the cerebrospinal fluid, lung, lymph tissue, spleen, kidney, liver, and ileum. Studies have shown that transfusion of blood from sheep infected with either BSE or scrapie but without evident disease can infect naive sheep. Although the risk of cross-contamination is always present, to date no studies have shown that blood can transmit disease from cattle with BSE. Embryos from BSE-affected cattle have not transmitted diseases to mice. Calves born of dams that received embryos from BSE-affected cattle have survived for up to 7 years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy.

Detection Strategies

No currently available procedures have been validated as being sufficiently sensitive for routine antemortem screening of asymptomatic animals, although analytical methods are under development for detection and quantitation from low-infectivity materials such as blood. The classic diagnostic test for TSEs is postmortem histological examination of brain tissue to confirm characteristic vacuolar degeneration. Other testing options include immunohistochemical tests that can confirm the presence of PrPSc, the abnormal disease-specific conformation of prion-related protein (PrP), in the vacuolated regions of the brain; and immunochemical tests such as Western blots and enzyme-linked immunosorbent assays that can detect PrPSc in tissues with high or moderately high titers. These tests typically take less time to perform than histological examination (6–8 hours vs. weeks, respectively) and can be partially or fully automated. Although most of these are postmortem tests, studies have demonstrated the feasibility of antemortem testing of lymphoid tissue samples from the tonsils or from the third eyelid of infected animals. Immunochemical tests require extensive sample collection and preparation and can be cost prohibitive for routine testing and monitoring the disease state of large herds. Diagnostic strategies must consider the sensitivity of testing in certain tissues as well as the test's ability to detect infectivity in animals before the development of clinical signs of disease. Negative results do not ensure the absence of infectivity. Detection of infectivity is possible if suspect tissue is inoculated into experimental animals

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intracranially where the causative agent can amplify. This approach for detection of low infectivity can take months to years to yield a positive result.

Risk Assessment and Risk Reduction Strategies

Serum manufacturers should employ risk reduction strategies to eliminate the danger of cross-contamination of fetal blood with other tissues, including appropriate sourcing of animal-derived articles and using practices that have been shown to eliminate or minimize the risk of transmitting TSE, via either foods or health care products. Serum end users should perform a risk assessment of their sourcing strategy that takes into account the amount of bovine serum used in their application and should conduct supplier audits to ensure traceability of sourcing, handling, and appropriate quality control systems.

SOURCE AND AGE OF ANIMALS

Serum manufacturers should monitor the traceability of each lot of serum to ensure the qualification of bovine serum, as described previously in the two sections *Serum Harvesting and Processing* and *Quality Control*. In addition to traceability, careful selection of source materials is the most important criterion for the safety of medicinal products. Certification of the origin must be available from the supplier, and manufacturers should keep this information on file. The U.S. Food and Drug Administration (FDA) recommendations prohibit the use in FDA-regulated products (except gelatin) of any bovine-derived materials that originate from countries that report indigenous cases of BSE. The current proposed rule qualifies FBS as an unlikely source of BSE infectious material, because current evidence suggests that cow-to-calf transmission of BSE is unlikely. The proposed rule also states that prohibited cattle materials do not include materials sourced from fetal calves of cows that were inspected and passed, as long as the materials were obtained by procedures that can prevent contamination with specified risk materials. For veterinary biologics, current regulations enforced by the USDA's Center for Veterinary Biologics (CVB) indicate that ingredients of animal origin should be sourced from countries with no or low BSE risk, as defined by the U.S. National Center for Import and Export and 9 CFR 94.18.

The most satisfactory sources of materials are from countries with the following:

- No reported cases of indigenous BSE
- Compulsory notification of positive tests
- Compulsory clinical and laboratory verification of suspected cases
- Prohibition of the use in ruminant feed of meat and bone meal containing any ruminant protein
- No importation of cattle from countries where a high incidence of BSE has occurred
- No importation of progeny of affected females

BSE infectivity may increase with animal age. Although bovine serum is considered a low-risk material for TSE transmission, some end users consider it prudent to source serum from dams below a set maximum age. If manufacturers cannot determine the date of the dam's birth, they should consider both the implementation date of the feed ban in the country of origin and the incubation period of BSE in order to determine the safety of the source. A ruminant feed ban was imposed in the United Kingdom in July of 1988. These considerations are lot specific, so audits of the raw material supplier should include a review of records.

PRODUCTION PROCESS

End user manufacturing systems should be in place for monitoring the production process and for batch delineation (definition of batch, separation of batches, and cleaning between batches). Of primary importance is control of the potential for cross-contamination with possible infectious material. Because of the documented resistance of TSE agents to most inactivation procedures, controlled sourcing is the most important criterion in achieving acceptable product safety.

Whenever possible, manufacturers should identify steps that theoretically or demonstratively remove or inactivate agents during the manufacture of the material. Manufacturers should continue their investigations into removal and inactivation methods to identify steps/processes that will help ensure the removal or inactivation of TSE agents. Manufacturers should design production processes using available methods that have the greatest likelihood of inactivating or removing TSE agents. For example, prolonged exposure of tissues to high moist heat and high pH inactivates the BSE agent. Such treatments, however, are inappropriate for the extraction of many other types of bovine-derived articles, such as serum, because these treatments lead to the destruction of the material. Conventional chemical and biochemical extraction and isolation procedures may be sufficient to remove the infectious agent. Similar techniques may be effective for other bovine-derived articles. Further research will help to develop an understanding of the most appropriate methodology for validation studies. Issues to consider during validation of a process for removal of TSE agents include the following:

- The nature of the spiked material and its relevance to the natural situation
- Design of the study (including scale-down approaches)
- Method of detecting the agent (in vitro or in vivo assay) after spiking and after the treatment
- Characterization and standardization of reference materials for spiking
- Data treatment and analysis (see *Design and Analysis of Biological Assays* ⟨111⟩)

Because no studies have successfully validated analytical methods for the detection of small amounts of the TSE agent, TSE clearance validation studies typically employ the intracranial injection of in-process material into rodents for amplification and detection of potential residual infectivity.

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TESTING AND CONTROL OF ADVENTITIOUS AGENTS

Introduction

Rigorous testing procedures, strict processing and production guidelines, and appropriate risk assessments help ensure the safety of the different types of bovine serum. This section discusses specific tests that can detect and control adventitious agents.

Adventitious Agents Testing

The adventitious agents testing required for the evaluation of master seeds, master cells, and bulk and final products is described in 9 CFR 113.53 and by directives from the European Agency for the Evaluation of Medicinal Products (EMEA) (EMEA/CVMP/743/00 and EMEA/CPMP/BWP/1793/02). The testing methods outlined in these documents can detect a wide range of bovine microbial agents in serum products. These testing methods meet the requirements for most of the world's regulatory agencies. Serum manufacturers should test a representative sample of each batch of serum to determine the presence of adventitious agents. Testing is performed after filtration but before any further processing that is intended to inactivate or remove viruses.

Filtration with 100-nm (0.1-µm) pore size filters is an accepted method for removing mycoplasmas and gamma irradiation (> 25 kGy while frozen), and chemical treatments (e.g., with betapropiolactone) are accepted methods of inactivating viruses and mycoplasmas; serum manufacturers routinely use these tools in both production and testing facilities. These treatments do not remove antibodies that may interfere with some applications. Additionally, the treatments do not ensure complete viral removal or inactivation, but can significantly reduce the risk of viral activity. The testing series to screen bovine serum for the absence of adventitious agents typically includes the following:

- Bacterial and fungal sterility testing as described in 9 CFR 113.26
- Mycoplasma testing as described in 9 CFR 113.28
- Viral testing as described in 9 CFR 113.53

The procedures described in *Sterility Tests* ⟨71⟩ confirm the absence of bacterial and fungal infection. For viruses, only cultivation using suitable substrate cells can indicate viral infectivity and replication. Those who use serum for research or production should test the serum for the absence of adventitious agents in a manner that is consistent with the product's intended application, bearing in mind that testing indicates only presence or absence of adventitious agents within the limits of the test procedures used.

Mycoplasma Testing

Mycoplasma contamination in tissue culture can arise from many animal origin sources, including serum, but more commonly it results from cross-contamination of infected cultures. Mycoplasmas are particularly insidious contaminants in cell culture because they

- cannot be visualized by light microscopy even at high density (>10^7 colony-forming units/mL);
- cause no observable change in turbidity or pH of the culture fluid;
- cannot routinely be removed by single sterilizing filters, although removal can be obtained through a triple series of 0.1-µm filters;
- are unaffected by traditional antibiotics used in cell culture; and
- exert an extremely wide variety of adverse effects in tissue culture.

Classical mycoplasma detection is described in *Mycoplasma Tests* ⟨63⟩.

In addition to these methods, more recent detection procedures include luminescent and polymerase chain reaction (PCR) assay procedures. *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩ describes the general principles of PCR assays. The sensitive 20-minute luminescent assay measures a specific enzyme activity of mollicutes that converts adenosine diphosphate to adenosine triphosphate via a luciferase/luciferin reaction. Results are unequivocal and semiquantitative. PCR methods are quick and sensitive and display with good reliability, but occasional false positive results are a source of concern with commercial testing service labs. PCR may detect mycoplasmal DNA fragments that are non-infectious.

Viral Testing

The virus testing procedures for serum products are outlined in 9 CFR 113.52 and 9 CFR 113.53. In addition, there are other documents that may include equivalent or relevant testing such as EMEA/CVMP/743/00-Rev.2 from the Committee for Veterinary Medicinal Products (CVMP) *Revised Guideline on Requirements and Controls Applied to Bovine Serum Used in the Production of Immunological Veterinary Medicinal Products* and EMEA/CPMP/BWP/1793/02 from the Committee for Proprietary Medicinal Products (CPMP) *Note for Guidance on the Use of Bovine Serum in the Manufacture of Human Biological Medicinal Products*. Serum manufacturers should perform virus testing in compliance with this regulation, using at least two different and sensitive detector cell lines, one of which should be of bovine origin. The tests include cultivation of detector cells in cell culture media supplemented with 15% test serum for at least 21 days. Cells are subcultured at least twice during this period, usually 7 and 14 days post inoculation. At the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are examined for general signs of virus amplification. The following end points are used for general virus detection: microscopic cell examination for cytopathogenic agents such as infectious bovine rhinotracheitis virus, cell staining and microscopic examination for inclusion bodies, and hemadsorption test to detect hemadsorbing agents such as PI-3. In addition to this series of testing and at the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are stained with specific fluorescent antibodies against the following specific viral agents:

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- BVDV
- Bovine parvovirus
- Bovine adenovirus
- Bluetongue virus
- Bovine respiratory syncytial virus
- Reovirus
- Rabies virus

In addition to the viruses listed above, other viruses can be causative agents of disease and may require testing in various bovine serum applications. The serum end user is responsible for determining whether full 9 CFR testing is sufficient, and if other specific viral agents should be tested for. Examples of specific viruses not covered by the current virus testing guide may include akabane, bovine herpesvirus 1 (BHV-1), Parainfluenza-3 virus (PI-3), bovine leukemia, bovine rotavirus, bovine circovirus, bovine polyomavirus, coronavirus, torovirus, bovine enterovirus, bovine astrovirus, foot-and-mouth disease virus (FMDV), and rinderpest. *Appendix 2* provides a general description of some of these viruses as well as the ones for which testing is required. A serum end user's thorough risk analysis should determine the scope of testing and serum treatment options.

Risk Assessment and Detection Strategies

Serum manufacturers and serum end users should carry out a comprehensive, science-based risk assessment (e.g. Failure Modes and Effects Analysis) in order to better understand the safety profile of the serum product. The following risk assessment elements can be taken into consideration, but other elements can be included as appropriate: country of origin, region of the country, animal disease status of the country/region of origin, animal age, blood collection process, animal stunning method and exsanguination method, serum manufacturing process, type of production quality system, production in-process controls, final product testing, virus inactivation, equipment segregation, equipment cleaning procedure, personnel training, serum use/application, pharmaceutical product type, and intended use.

The species barrier provides a degree of protection against infection by some animal etiologic agents. This barrier is not an alternative to proactively ensuring that pharmaceutical products are manufactured only from raw materials of animal origin that have undetectable levels of adventitious agents. Inoculation of viable organisms into a nonhost species carries a risk that the organisms could cross the species barrier. An appropriate test regimen of serum material should therefore include examination for potential contaminants associated with the species of origin and the species of intent. Serum treatments to inactivate viral agents are a factor in establishing the appropriate test regimen for a particular material. Lowest risk of contamination is associated with biological materials that are terminally sterilized.

Zero risk is neither possible nor reasonable. The serum manufacturers should fully describe specific testing regimens in the product specifications, and these will vary depending on the type and source of the serum. Therefore, the guidelines for screening described in this chapter are examples only, and screening for all viruses listed may not be required for a particular material. Some manufacturers may perform certain tests on the finished product or on in-process materials rather than on individual component(s). Manufacturers must also evaluate the dilution effect in relation to the limit of detection of the test procedure. Interference with growth or neutralization of viral activity by serum may be an indication of a specific antibody or certain nonspecific factors in serum masking the viral agent. It is recommended that serum manufacturers consider this possibility when determining an adequate level of treatment in their viral inactivation studies or in virus testing applications.

Serum manufacturers should confirm that the species of origin is bovine to ensure that no other nonbovine agents may be present. Manufacturers should perform extraneous virus testing in appropriate cell cultures (see *Virology Test Methods* ⟨1237⟩ for appropriate cell line choices dependent on assay and targeted agent). If necessary, seroconversion studies should be conducted in susceptible animal species using a host species immune antibody response as the method of detection. Studies should use this procedure following an inactivation step to detect whether the virus was present before the virus inactivation process.

Serum manufacturing processes should be conducted in a consistent manner, following the established manufacturing procedures, with adequate quality systems built into the production process. Furthermore, equipment segregation (by species of origin), equipment and facility cleaning procedures, and personnel training are important elements in the risk assessment of the process.

Safety Considerations

End users of donor bovine serum may require serum that does not have detectable antibodies against BVDV or other specific agents so that the users can propagate cell cultures used in vaccine production, diagnostic testing, and test kit preparation, especially for the maintenance of master seed and master cell stocks. More than 40 cell types are available for the production of veterinary biologicals, but fewer than 10 media types are available for their propagation. Some researchers have proposed serum-free media as an alternative in propagating certain cells and viruses; but this means adapting culture procedures, which may alter the cells and change production results. If new or different sera are imported into the U.S., serum end users will require confirmation of source, species, and documentation of the origin of the sera in countries that are free of FMD and rinderpest.

CHARACTERIZATION OF BOVINE SERUM

Introduction

In the absence of end product-specific requirements, each lot of FBS should be tested to confirm that the serum meets the requirements of the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩. For all other

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types of bovine sera, this section describes several key procedures for characterization. These procedures are not mandatory but are guidelines that manufacturers may consider for their individual applications. The table in the *Hemoglobin* section shows samples of specifications for the different types of bovine sera.

Species Identification

Both inter- and intraspecies identification assays should be performed on bovine sera to confirm species identity and the integrity of the serum products, and to ensure that nonbovine agents are not present. The most commonly used assay for the identification of bovine species identity is based on the electrophoretic profile of specific serum proteins. With electrophoresis, the serum proteins usually separate into as many as six fractions: albumin, alpha 1, alpha 2, beta 1, beta 2, and gamma globulins.

Other procedures used for bovine speciation include radial immunodiffusion (RID) and the double diffusion Ouchterlony method. These procedures allow either qualitative or quantitative measurements of the immunoglobulin G levels in serum. The RID method is based on the diffusion of an antigen from a circular well into a homogeneous gel that contains specific antiserum for each particular antigen. A circle of precipitated antigen and antibody forms and continues to grow until it reaches equilibrium. The diameters of the rings are a function of antigen concentration. The Ouchterlony method is a double gel diffusion test wherein antigen and antibody diffuse toward each other in a semisolid medium to a point in the medium where optimum concentration of each is reached, forming a precipitate. The Ouchterlony plates contain cylindrical wells—a central 8-mm diameter antigen well, surrounded by six 3-mm antisera wells—which make possible the simultaneous monitoring of multiple antigen–antibody systems and the identification of particular antigens in a preparation. The proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩ describes the accepted procedure.

Hemoglobin

Hemoglobin is a multi-subunit protein that forms an unstable reversible bond with oxygen in the red blood cells. The oxygen-loaded form is called oxyhemoglobin and is bright red. The oxygen-unloaded form is called deoxyhemoglobin and is purple-blue. Oxyhemoglobin is the predominant form in red blood cells.

Low hemoglobin content in sera is widely accepted as a good general indication of rapid and careful processing of blood that will be used for serum. Red blood cells are fragile and rupture easily, releasing hemoglobin into the serum. Rough handling of the harvested blood, poor temperature control, or delayed processing elevates hemoglobin content in serum. Acceptable levels of hemoglobin may vary with intended application. The hemoglobin levels are measured using spectrophotometric procedures (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩) as described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩.

	FBS	Newborn Calf Serum	Calf Serum	Donor Bovine Serum	Adult Bovine Serum
Sterility test	No growth detected	No growth detected	No growth detected	No growth detected	No growth detected
Mycoplasma	Not detected	Not detected	Not detected	Not detected	Not detected
Virus testing	Not detected	Not detected	Not detected	Not detected	Not detected
Hemoglobin (mg/dL)	<30	<30	<30	<30	<30
Total protein (g/dL)	3.0–4.5	3.5–6.0	5.0–8.0	5.0–8.0	6.0–10.0
pH	7.00–8.00	7.00–8.00	7.00–8.00	7.00–8.00	7.00–8.00
Osmolality (mOsmol/Kg)	280–360	240–340	240–340	240–340	240–340

Chemical Profile

The testing of components such as cholesterol, alpha globulin, beta globulin, gamma globulin, albumin, creatinine, bilirubin, glucose, alanine aminotransferase, aspartate aminotransferase, phosphorus, potassium, calcium, and sodium usually is not considered a criterion for bovine serum lot release. Some manufacturers do not perform the tests on a routine basis but only as auxiliary tests. In some instances hospital clinical laboratories may run the tests. The levels of these chemicals in serum are important to end users and may also be used to assess lot-to-lot variability.

Endotoxin Levels

Although high endotoxin levels are not suitable for applications involving injectables, acceptable levels in bovine sera vary depending on the intended application. Some manufacturers may overlook the importance of low endotoxin levels in bovine sera used in cell culture applications. Endotoxin influences more than 30 biological activities. Some of these are macrophage activation, mitogenic stimulation, and induction of interferon and colony-stimulating factor (for some applications, these may be positive activities). Endotoxin can also lead to cytotoxicity by initiating complement activation. The most commonly used methods for endotoxin detection are the semiquantitative gel clot Limulus amebocyte lysate procedure and the quantitative kinetic chromogenic method described in *Bacterial Endotoxins Test* ⟨85⟩. For both the gel clot and the kinetic chromogenic assays, valid endotoxin assays require appropriate treatment by heat or dilution in order to avoid adverse effects of interfering substances in serum. Researchers should include a positive product control in each assay to confirm that any interference has been overcome by the heat or dilution treatment.

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Osmolality

The osmolality test is designed to evaluate the electrolyte concentration in bovine serum. Chemicals that affect serum osmolality include sodium, chloride, bicarbonate, potassium, proteins, and glucose. Serum manufacturers should measure the osmolality of each serum batch to verify compliance with product specifications, using equipment calibrated with standards that are traceable to the National Institute of Standards and Technology. *Osmolality and Osmolarity* ⟨785⟩ describes how osmolality is determined by freezing-point depression of the bovine serum solution. Scientists use at least two standards to calibrate the instrument. The osmolality of each sample is calculated and related to the serum water content and is expressed as mOsmol/kg H_2O.

Total Protein Level

The total protein level in serum is measured to verify animal age and compliance with product specifications. *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩ describes two procedures, the Biuret and Bradford methods, for determining protein concentration. The acceptable level of protein in serum should be assessed by the end user based on the intended application.

Cell Growth Properties

Each lot of serum should be tested for its ability to support in vitro growth of specific cell lines. Bovine sera are highly variable, and different lots may yield different results. Because of this variability, end users should characterize and standardize the cell lines that they will use for this type of testing. End users should design cell growth procedures that will help them check the efficacy of bovine serum in promoting cell growth. Serum manufacturers will benefit from monitoring growth promotion over several generations of subcultures to detect any evidence of cytotoxicity or changes in cell morphology. Different serum manufacturers use different cell types, and the growth studies and cell lines used by serum manufacturers also may differ from those applied by serum end users. When serum manufacturers evaluate the growth properties of a specific cell line in response to a specific lot of serum, they should take into account plating efficiency and/or growth promotion or some other functionality tests that qualify the serum lot for its intended use.

Plating efficiency at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This procedure can reveal differences in the growth rate within the population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). The growth kinetic is another important aspect in the design of cell-based experiments. Determining the growth curve of each cell line helps define optimal culture conditions, because variation in serum and other growth additives may influence growth parameters, which may affect the experimental outcome.

In the absence of specific tests designed for their particular products, serum users can refer to the functionality tests described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩ to determine whether a lot of serum is suitable for their application. This chapter provides guidance about how to perform growth promotion and plating efficiency tests.

In Vitro Cytotoxicity

Serum manufacturers should use an appropriate cell line for testing each lot of serum, and should perform growth studies through several subcultured generations to ensure that the serum has no cytotoxic effect on the cells. The choice of cell line depends on the intended use of serum. The cell growth and cytotoxicity assays should be performed on the final batch of serum after any viral inactivation step or any further processing.

CONCLUSION

Bovine serum is likely to remain an important component in the manufacture of many biologics, particularly those relying on cell culture. As with similar materials, bovine serum displays inherently variable quality. As a result, serum end users must establish suitable tests, procedures, and acceptance criteria for introduction of materials into a particular application process that uses serum. This may mean screening multiple lots of bovine serum to determine which lots meet the specification (see the section *Characterization of Bovine Serum*).

Manufacturers of therapeutic products using bovine serum are responsible for ensuring and documenting its quality and its impact on the quality, safety, and efficacy of the final product. In addition, it is important to ensure that each lot of serum performs in an equivalent manner during manufacturing. Serum can also interfere with final product purification; therefore it is important to understand the effect of bovine serum on the manufacturing process in order to understand the effect that various processes might have on the final product. Finally, risks can also be mitigated through the design of processes to include steps to adequately remove the bovine material through dilution, separation, or inactivation as well as the development of analytical assays to assess the bovine-derived residual content during processes and in the final therapeutic product. A number of sensitive assays can provide a quantitative means of detecting bovine material at picogram levels.

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APPENDICES

Appendix 1: Relevant Regulatory References

Bovine sera and serum-related products used in the manufacture of biological products are regulated in the context of *Requirements for Ingredients of Animal Origin Used for the Production of Biologics*, 9 CFR 113.53. Currently, individual serum manufacturers perform detection studies to identify contaminating viruses. Because of the potential international market for serum, serum manufacturers need to be mindful of other regulatory requirements. Manufacturers can use the documents listed here as guidance for screening bovine sera for contamination by adventitious agents. Because of the risk carried by animal-derived serum products, serum manufacturers and end users should ensure that the country of origin of the material complies with applicable regulatory requirements. Although no cell performance assays currently demonstrate lack of BSE in serum, serum manufacturers must comply with the regulatory requirements of countries where the serum is sourced and marketed to ensure a minimal risk of infection with BSE/TSE.

Beyond relevant *USP* chapters referenced in this chapter, the following list of documents includes regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance.

CFR

- 9 CFR 94.18 (CVB, 2001)
- 9 CFR 113.46
- 9 CFR 113.47
- 9 CFR 113.52
- 9 CFR 113.53
- 9 CFR 113.55
- 9 CFR 320
- 9 CFR 327.4
- 21 CFR 211 Subpart E
- 21 CFR 801.1
- 21 CFR 809.10

FDA

- FDA. Center for Biologics Evaluation and Research (CBER). 2000. Letter to manufacturers of biological products. Available at: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ucm105877.htm
- Use of materials derived from cattle in medicinal products intended for use in humans and drugs intended for use in ruminants (Proposed Rule). *Federal Register.* 2007; *72*(8): 1582–1619. Available at: http://www.reginfo.gov/public/

International Regulations and Guidance Documents

- CPMP/Biotechnology Working Party/EMEA (CPMP/BWP/EMEA). 1996. *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses*. Available at http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003684.pdf.
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Appendix 2: Viruses to Consider when testing Bovine Serum

Following is a general description of viruses that manufacturers can consider when testing bovine serum for the absence of adventitious agents. The list is intended only to provide general information. The list of required testing is described in this chapter in the section *Viral Testing*.

AKABANE

An insect-transmitted virus that causes congenital abnormalities of the central nervous system in ruminants. Disease due to *Akabane* virus has been recognized in Australia, Israel, Japan, and Korea. Antibodies to it have been found in a number of countries in Southeast Asia, the Middle East, and Africa. The disease affects fetuses of cattle, sheep, and goats. Asymptomatic infection has been demonstrated serologically in horses, buffalo, and deer (but not in humans or pigs) in endemic areas.

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Bluetongue

An infectious, noncontagious arthropod-borne viral disease primarily of domestic and wild ruminants. Infection with bluetongue virus is common worldwide but is usually subclinical or mild. *Bluetongue* virus is the type-species of the genus *Orbivirus* in the family Reoviridae. Worldwide, 24 serotypes have been identified, although not all serotypes exist in any one geographic area: e.g., only 5 serotypes (2, 10, 11, 13, and 17) have been reported in the U.S. Distribution throughout the world parallels the spatial and temporal distribution of vector species of *Culicoides* biting midges, which are the only significant natural transmitters of the virus.

Bovine adenovirus

Associated with a wide spectrum of diseases. *Bovine adenovirus* type 3 is the serotype most often associated with bovine respiratory disease. *Bovine adenoviruses* are DNA viruses that have been separated into two genera: the *Mastadenovirus,* or *mammalian adenoviruses,* and the *Aviadenovirus,* or *avian adenoviruses.* Within the genus *Mastadenovirus* are numerous species-specific serotypes, nine of which have been identified in cattle. *Epitheliotrophic adenoviruses* have also been isolated from ruminants, and usually are clinically unapparent. Clinical disease is dictated by various factors, including the strain of virus, concurrent infection, stress, environmental conditions, and management practices.

Bovine herpesvirus 1 (BHV-1)

Associated with several diseases in cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, conjunctivitis, abortion, encephalomyelitis, and mastitis. BHV-1 infections are widespread in the cattle population. In feedlot cattle the respiratory form is most common.

Bovine leukemia

An exogenous C-type oncovirus in the family Retroviridae. Bovine leukemia is a viral disease of adult cattle characterized by neoplasia of lymphocytes and lymph nodes. Infection occurs by iatrogenic transfer of infected lymphocytes and is followed by a permanent antibody response. The prevalence of infection in a herd may be high, but only a few animals develop fatal lymphosarcoma. Infection is spread by contact with contaminated blood from an infected animal.

Bovine reovirus

Double-stranded ribonucleic acid (RNA) (dsRNA) viruses with nonenveloped spherical virions 60–80 nm in diameter. They cause bovine respiratory diseases.

Bovine respiratory syncytial virus (BRSV)

An RNA virus classified as a pneumovirus in the Paramyxovirus family. This virus was named for its characteristic cytopathic effect—the formation of syncytial cells. In addition to cattle, sheep and goats can also be infected by respiratory syncytial viruses. Human respiratory syncytial virus (HRSV) is an important respiratory pathogen in infants and young children. HRSV has antigenic subtypes, and preliminary evidence suggests the existence of antigenic subtypes of BRSV. BRSV is distributed worldwide, and the virus is indigenous in the cattle population. BRSV infections associated with respiratory disease occur predominantly in young beef and dairy cattle.

Bovine rotavirus

A dsRNA spherical virion 60–80 nm in diameter without an envelope. It is the most common viral cause of diarrhea in calves and lambs.

Bovine viral diarrhea virus (BVDV)

An RNA virus classified as a *Pestivirus* in the family Flaviviridae. BVDV can cross the placenta and appears to be capable of inducing immunosuppression, which allows the development of secondary bacterial pneumonia. BVDV has been reported to be the virus most frequently associated with multiple viral infections of the respiratory tract of calves.

Foot-and-mouth disease (FMD)

A highly infectious viral disease of cattle, pigs, sheep, goats, buffalo, and artiodactyl wildlife species. In a susceptible population, morbidity approaches 100%. The disease is rarely fatal except in young animals. FMD is caused by an *Aphthovirus* of the family Picornaviridae. Seven immunologically distinct serotypes are known, and within each serotype exist a large number of strains that exhibit a spectrum of antigenic characteristics.

Parainfluenza-3 virus (PI-3)

An RNA virus classified in the Paramyxovirus family. Although PI-3 is capable of causing disease, the virus usually is associated with mild to subclinical infection. The most important role of PI-3 is to serve as an initiator that can lead to the development of secondary bacterial pneumonia. Infections caused by PI-3 are common in cattle.

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PARVOVIRUS

A relatively heat-stabile single-stranded DNA virus approximately 20 nm in diameter that has been recovered from cattle but under natural conditions is not known to cause disease.

RABIES

An acute viral encephalomyelitis that principally affects carnivores and bats, although it can affect any mammal. Rabies is caused by *Lyssaviruses* in the Rhabdovirus family. Although they are usually confined to one major reservoir species in a given geographic area, spillover to other species is common.

RINDERPEST

A *Morbillivirus,* closely related to the viruses that cause canine distemper and measles. Strains may vary markedly in host range and virulence. Sera from recovered or vaccinated cattle cross-react with all strains in neutralization tests, but minor antigenic differences have been demonstrated. The virus is fragile and becomes rapidly inactivated by heat and light but remains viable for long periods in chilled or frozen tissues. Rinderpest is endemic in many countries in Asia and Africa. Historically, the virus has been widely distributed throughout Europe and Africa but to date has not established itself in North America, Central America, the Caribbean Islands, South America, Australia, or New Zealand. Rinderpest is included in the WHO's Office International des Epizooties list of communicable diseases that have the potential for very serious and rapid spread, irrespective of national borders; that are of serious socioeconomic or public health consequence; and that are of major importance in the international trade of livestock and livestock products.

⟨1025⟩ PANCREATIN

OUTLINE

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1. INTRODUCTION

Pancreatin is a pancreatic enzyme preparation containing amylase, protease, and lipase enzymes isolated from the pancreas gland of the hog, *Sus scrofa* L. var. *domesticus* Gray (Fam. Suidae). The pancreas is a secretory organ that plays a crucial role in the digestive process by producing bicarbonate to neutralize the acidic environment in the duodenum, hormones to regulate various catabolic functions, and a variety of digestive enzymes to degrade food in the small intestine. Pancreatin and pancreatin-containing medicinal products are used to aid digestion and absorption of food (carbohydrates, fat, and proteins) in patients with exocrine pancreatic insufficiency (EPI) caused by cystic fibrosis, chronic pancreatitis, and other conditions that might cause a deficiency in the secretion of pancreatic enzymes.

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Pancreatic enzyme products (PEPs) of porcine origin have been marketed in the United States for the treatment of EPI before the enactment of the Federal Food, Drug, and Cosmetic Act of 1938. Supplemental pancreatic enzymes are available in prescription and non-prescription forms. Since 2004, the U.S. Food and Drug Administration (FDA) has required that all pancreatic enzyme drug products marketed in the United States obtain FDA approval via a New Drug Application (NDA). Although over-the-counter pancreatic enzyme products are available without a prescription, they are classified as dietary supplements rather than drugs. Pancreatin and pancrelipase share similar functions and indications; however, pancrelipase, which is available only as a prescription drug, contains more of the active lipase enzyme and also more purified pancreatic extract than pancreatin does.

This chapter describes best practices related to the sourcing and manufacturing of pancreatin raw materials used in both pancreatin and pancrelipase drug products; these best practices help to ensure the safety and efficacy of the drug products made from this active pharmaceutical ingredient (API). A list of applicable regulatory guidance documents is provided in *Appendix 1: Regulatory Bibliography*.

2. PANCREAS COLLECTION AND PANCREATIN PRODUCTION

The animal-sourced raw material (pancreas glands) intended for pharmaceutical processing is a by-product of meat production and is collected in slaughterhouses that are approved by the national competent authority and inspected by the relevant veterinary authority. The animals from which pancreatin is derived must fulfill the requirements for the health of animals suitable for human consumption.

An example of a typical manufacturing process is summarized in the description and flow chart (*Figure 1*) below.

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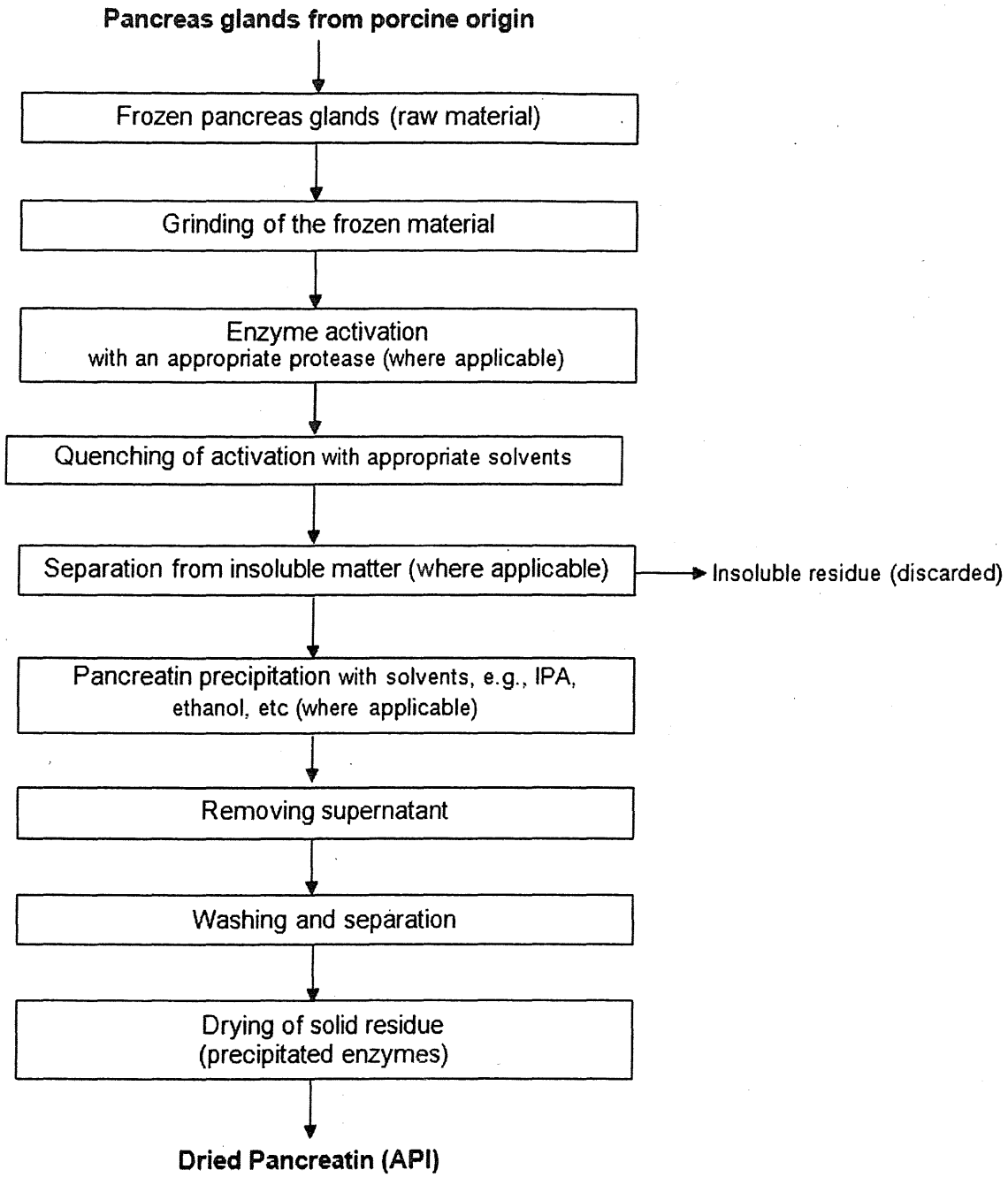

Figure 1. Flow chart showing the steps in the manufacturing process for pancreatin.

The pancreatic glands should be kept frozen to prevent a loss of enzymatic activity during holding and transport. After the manufacturer accepts the frozen materials, the pancreatin is extracted and purified under conditions that reduce microbiological load and other potential impurities. To accomplish this, the frozen pancreatic glands are macerated into fine slurry and treated with activators to convert the inactive pancreatic enzyme precursors (zymogens) into active enzymes. The activation is carried out under controlled conditions; factors such as time, temperature, ionic strength, or concentration are controlled as defined by each manufacturer's process. Once the activation is complete, the activation step is stopped by the addition of solvents such as acetone, isopropyl alcohol, or other enzyme-compatible solvents. After separation from insoluble matter (where applicable), the mixture is combined with solvent to precipitate the pancreatin. The supernatant is separated, and the solid residue is washed with solvent. Finally, the pancreatin is dried at appropriate temperature and vacuum. The biological activity may be adjusted

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and/or stabilized by adding suitable fillers, such as lactose; sucrose containing not more than 3.25% starch; pancreatin of lower digestive power; microcrystalline cellulose; maltodextrin; or sodium chloride.

3. QUALITY CONTROL

Each lot of pancreatin is subject to appropriate quality control testing. Quality control testing needs to address the requirements of the applicable monograph, as well as other identified quality parameters. Such tests should include appearance, identification, purity, and activity of the pancreatin. Process- and product-related impurities, such as residual solvents from extraction and precipitation and fat, should be considered.

Water is critical for enzyme activity and stability, and thus the limit of water content should be specified and monitored by appropriate analytical methods such as loss on drying (LOD) (see chapter ⟨731⟩ *Loss on Drying*). The activity assays are applied to confirm that pancreatin meets predefined limits for activity levels of lipase, amylase, and proteases, which are considered critical quality attributes.

Because pancreatin is of biological origin, quality control includes microbial testing as well as testing for the absence of certain adventitious agents that are pathogenic to humans.

4. LABELING

Product should be labeled in conformance to monograph and regulatory requirements but also should conform to customer requirements as applicable. The label contains information such as the name and address of the manufacturer, manufacturing date, retesting date, expiration date, lot number, storage conditions and specific precautions (e.g., "protect from moisture"), and a statement indicating the intended use.

5. CERTIFICATION AND DOCUMENTATION

The product must be accompanied by required certificates and documentation, as applicable. This documentation should include a certificate of analysis (COA) or other certificate documenting conformance to monograph requirements and quality control test results; a certificate of origin indicating the animal species; and certifications related to adventitious agents, including, where applicable, Transmissible Spongiform Encephalopathy/Bovine Spongiform Encephalopathy statements. Other pertinent and required information, such as retest or expiration date, storage, and packaging recommendations, should also be included as applicable.

The manufacturer/supplier should also be able to provide documentation for regulatory purposes containing the following information:

- Information on the regulatory agency's approval/license number and the full address of the pancreatin manufacturing site.
- A statement that the animal-sourced raw materials intended for pharmaceutical processing were collected and delivered under oversight of the responsible/competent authorities.
- A list of the countries of origin of the collected raw materials.
- A statement that during transportation, the pancreas glands can be identified for animal by-products intended for pharmaceutical purposes by appropriate documentation according to the corresponding legal regulations (such as animal health certificates from official veterinarians or a technical trade documentation).
- A statement that the pancreas glands are collected in approved slaughterhouses, and the animals from which pancreas glands are collected have undergone inspection in compliance with the current applicable legislation and were declared as suitable for human consumption, or no visible signs of diseases transmittable to humans or animals were detected at the time of slaughter.

6. TESTING AND CONTROL OF ADVENTITIOUS INFECTIOUS AGENTS

6.1 Overview

Although there have been no reports documenting any infectious illness subsequent to the use of pancreatin-derived medicinal products, there is a theoretical risk of porcine pathogen transmission from pancreatin.

The safety of porcine-derived PEPs should be enhanced by implementation of multiple complementary and/or overlapping strategies for adventitious agent containment, clearance, and control. Manufacturers should take an ongoing, risk-based approach to enhancing the safety of these products with regard to adventitious agents that includes incorporation of risk assessment, risk mitigation, and process materials management strategies. Testing should be included, when appropriate, to provide maximum possible assurance of a sufficiently low risk of harm that is significantly outweighed by the therapeutic benefits and concurrent assurance of availability of the drug to patients.

6.2 Risk Assessment Strategies

The FDA's "Guidance for Industry: Exocrine Pancreatic Insufficiency Drug Products" encourages a risk-based approach to the potential for viral contamination of pancreatic enzyme products, in agreement with ICH Q5A(R1) and chapter ⟨1050⟩ *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*. The adventitious-agent risk profile of biological products in general is contingent upon a variety of factors including the origin of the biological, the type of raw

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materials used, manufacturing processes, and the route of administration. As sourcing and manufacturing may vary between manufacturers, each manufacturer should establish and implement an individual, full adventitious-agent risk assessment.

Although the scope of ICH Q5A(R1) covers the viral safety evaluation of biotechnology products derived from cell lines of human and animal origin, the principles and risk assessment approaches can provide the basis for a risk evaluation strategy for the pancreatin products. Applying the principles of ICH Q5A(R1), the risk minimization strategy to protect patients against inadvertent adventitious agent exposure should reflect a combination of three components:

1. **Sourcing:** Use of diligent sourcing to limit adventitious agents' access to the manufacturing process. Because pancreatin material is a by-product of the meat industry, it is important to ensure that pancreatin API is produced only from animals suitable for human consumption.
2. **Clearance:** Incorporation of robust clearance steps into the manufacturing process. The efficacy of these strategies depends on the adventitious agents' resistance to the type of physical and chemical inactivation used.
3. **Testing:** The control and testing of adventitious agents at suitable stages of the manufacturing process to provide assurance that any remaining load of potentially harmful adventitious agent is at sufficiently low levels. This is accomplished by using suitable screening assays against a relevant test virus panel. In this part of the risk assessment, the potential of porcine viruses to pose a risk to humans should be taken into account.

To identify the potential adventitious agents that might be present in a pancreatin preparation, manufacturers should identify adventitious agents that are present in the pig and assess the probability of their presence in the starting materials and API. Chapters ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* and ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* address testing for specific microbiological contamination, as defined in the relevant pancreatin-related product monographs. Additionally, manufacturers should specifically evaluate the potential presence of porcine viruses. In this evaluation, the manufacturing steps capable of removing or inactivating viruses should be identified, and the efficiency of the process for removing and/or inactivating viruses should be demonstrated by viral validation studies that follow applicable guidelines. Once a list of critical viruses potentially present in the starting material and/or in the API is established, manufacturers will decide on the appropriate process stages for viral testing and the level of viral testing to be performed on a batch-to-batch basis. Tests for specific viruses should be developed and validated, and acceptance criteria should be established and used in making accept/reject decisions about pancreatin API batches.

The potential zoonotic or non-zoonotic character of the virus should be taken into account when setting acceptance criteria.

6.3 Identification of Relevant Virus Panel Test

The risk assessment strategy described above requires the identification of potential viral contaminants of porcine-derived starting materials. *Table 1* gives an overview of enveloped and non-enveloped viruses known to be present in pigs that may present a contamination risk when using pigs deemed fit for human consumption as source animals. The risk assessment should address at least the viruses listed; however, depending on the origin of animals and the manufacturing process capability, the list may be adapted, and additional viruses may be considered (see the example in *Figure 2*). Manufacturers should implement systems to identify emergence of potentially relevant new viruses.

Table 1. Hazard Identification: Viruses Known to be Present in Pigs

Enveloped Viruses	Non-Enveloped Viruses
Classical swine fever virus	Encephalomyocarditis virus (EMCV)
African swine fever virus (ASFV)	Swine vesicular disease virus (SVDV)
Influenza virus	Foot and mouth disease virus (FMDV)
West Nile virus (WNV)	Reoviruses (REO)
Vesicular stomatitis virus (VSV)	Swine hepatitis E virus (swHEV)
Eastern equine encephalitis virus (EEEV)	Porcine rotaviruses (pRotaV)
Rabies virus (RABV)	Porcine enteroviruses (PEVs)
Porcine reproductive and respiratory syndrome virus (PRRSV)	Porcine parvoviruses (PPV)
Transmissible gastroenteritis virus (TGEV)	Porcine circovirus types 1 and 2 (PCV1/2)
Pseudorabies virus (SuHV-1)	
Nipah virus	
Porcine endogenous retroviruses (PERVs)	

6.4 Virus Clearance by Manufacturing Process Steps

Demonstration of viral clearance is a critical component of ensuring the overall safety of pancreatin-derived products. The objective of virus-clearance evaluation studies is not only to evaluate the ability of the manufacturing process to clear known viral contaminants and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process but also to estimate the capability of the manufacturing process steps to clear viruses in general. Viral clearance studies for the manufacturing process of pancreatin should be performed in accordance with the applicable, current guideline, ICH Q5A(R1).

Current pancreatin production processes are considered to be effective for inactivating enveloped viruses, following the results of virus clearance studies. However, non-enveloped viruses are more resistant to physico-chemical inactivation, making

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their inactivation more variable. Examples of viruses that are classified as having a high viral resistance toward treatment and that show moderate to limited inactivation include PPV and PCV1/2 (see *Figure 2*).

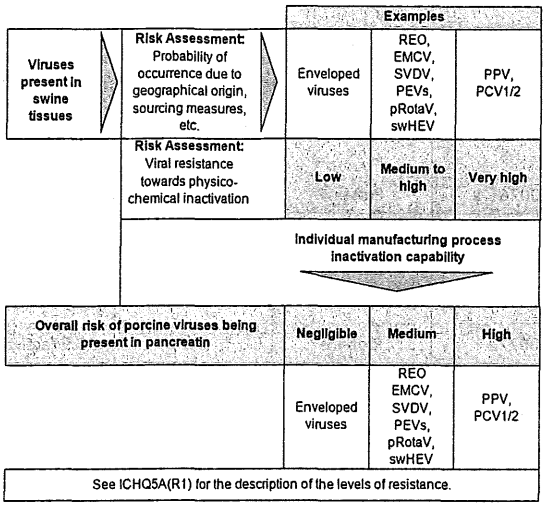

Figure 2. Example of a risk-assessment approach to virus identification for the test panel.

6.5 Viral Testing

A testing strategy is needed when the ability of the process to remove or inactivate a specific virus to appropriate levels has not been demonstrated. The potential zoonotic or non-zoonotic character of the virus should be taken into account when setting acceptance criteria, in terms of both assay sensitivity and specification limit setting. API batches that test positive for zoonotic viruses should be rejected. As a part of quality control, viral testing should be performed on each lot of API. The tests used should allow exclusion of any detectable load of potentially harmful levels of adventitious agent. The 9 CFR describes testing requirements for porcine biological products including live virus vaccines and antibody products but does not specifically address pancreatin. Applying the principles of 9 CFR, assays to detect porcine viruses can, for example, be based on cell culture monitoring for cytopathogenic effects for an appropriate incubation time, hemadsorption testing, virus-specific staining techniques, or appropriate combinations thereof (also see chapter ⟨1237⟩ *Virology Test Methods*). In addition to the technologies and viruses covered by 9 CFR, new molecular biology-based technologies may be used, and other viruses with zoonotic potential that are identified may require testing. Examples of specific viruses not covered by the current virus testing guide may include swHEV.

The definition of the test virus panel, selection of the appropriate process stage, suitability of the test method, and test method sensitivity should be justified by the manufacturer. All tests for specific viruses should be developed and validated in compliance with current guidance, for example, chapters ⟨1225⟩ *Validation of Compendial Procedures* and ⟨1033⟩ *Biological Assay Validation*, as applicable, and acceptance criteria should be established and used for accept/reject decisions of pancreatin API batches.

7. CHARACTERIZATION OF PANCREATIN

7.1 Description and Physico-Chemical Properties

Pancreatin is a slightly brownish to tan amorphous powder with a raw meat odor and taste. Pancreatin is partly soluble in water, forming a weak turbid solution, and insoluble in alcohol and ether. The following conditions may degrade pancreatin: mineral acids, alkali hydroxides, oxidizing agents, many metallic salts, and high humidity and temperatures.
Pancreatin-containing solutions should be filtered with caution, due to potential retention of the lipase and proteases on the filter. The enzymatic activity reaches a maximum in neutral-to-weakly alkaline solutions. The activity decreases quickly in acidic or strong alkaline solutions. The same applies to boiling of pancreatin-containing aqueous solutions. In non-enteric-coated formulations, exposing the product at a pH of 4.5 or less is not recommended, because a nearly complete loss of lipolytic activity has been observed.

Because pancreatin is of biological origin, other components are present in addition to enzymatically active proteins. These other components include proteins, amino acids, peptides, nucleic acids and fragments thereof, tissue components, fat, and inorganic substances. These components may have an impact on the quality of the final material.

7.2 Protein and Enzyme Contents

Pancreatin contains different digestive enzymes, most of which are produced and stored as zymogens (inactive precursors) in the pancreatic acinar cells. Under physiological conditions, pancreatic zymogens are transformed into active enzymes once the pancreatic secretion reaches the upper small intestine. An intestinal protease, enterokinase, triggers the activation process

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by cleaving the zymogen of trypsin, which further activates the other proteases. During the production process, the enzymes present in pancreatin are activated by trypsin (see *Figure 1*).

Table 2 summarizes some of the important characteristics of the main pancreatic enzymes found in pancreatin.

Table 2. Characteristics of Known Enzymes Present in Porcine Pancreatin

Name	E.C. Number	UniProtKB/ Swiss-Prot Accession Number	Substrates	Produced as a Precursor	Molecular Mass (kDa)	Sequence Length	Isoelectric Point
Trypsin	3.4.21.4	P00761.1	Proteins	Yes	23.8 (zymogen), 23.5 (activated)	231 a.a. (zymogen), 223 a.a. (activated)	9.3 (zymogen), 10.5 (activated)
Chymotrypsin	3.4.21.1	G1ARD6_PIG	Proteins	Yes	29.1 (zymogen), 25.6 (activated)	268 a.a. (zymogen), 221 a.a. (activated)	8.7
Elastase	3.4.21.36	P00772.1	Proteins	Yes	25.9 (activated)	250 a.a. (zymogen), 240 a.a. (activated)	8.5
Carboxypeptidase A1	3.4.17.1	P09954	Proteins	Yes	34.7 (activated)	308 a.a. (activated)	Not known
Carboxypeptidase B	3.4.17.2	P09955.5	Proteins	Yes	34.7 (activated)	305 a.a. (activated)	6.0
Kallikrein, glandular	3.4.21.35	P00752.4	Proteins	Yes	25–28 kDa	246 a.a. (zymogen), 239 a.a. (activated)	4.2–4.3
Triacylglycerol lipase	3.1.1.3	P00591.2	Triglycerides, diglycerides	No	50.1 (two glycosylation isoforms, lipases A and B)	450 a.a. (mature)	4.9 (lipase A), 5.0 (lipase B)
Colipase		P02703.3	No enzyme activity, cofactor of pancreatic lipase	Yes	10.3 (porcine procolipase A), 10.1 (porcine procolipase B)	93 a.a. (procolipase A), 95 a.a. (procolipase B)	Not known
Phospholipase A2	3.1.1.4	P00592	Phospholipids	Yes	14.7 (zymogen), 14 (activated)	146 a.a. (zymogen), 123 a.a. (activated)	4.4–4.5
Cholesterol esterase, also named carboxyl ester lipase (CEL), carboxyl ester hydrolase (CEH) or bile-salt stimulated lipase (BSSL)	3.1.1.13, 3.1.1.1	Complete amino acid sequence still unknown in pig	Cholesterol esters, vitamin esters, monoglycerides, phospholipids, galactolipids, some activity on triglycerides	No	Inconsistent values in the literature ranging from 65 to 98 kDa. While proteolytic forms have been identified, the exact mass is still unknown.	Complete amino acid composition still unknown in pig	4.2–4.8
α-Amylase	3.2.1.1	P00690.3	Polysaccharides	No	55.3	496 a.a. (mature)	5.95 (amylase I), 5.45 (amylase II)

7.2.1 PANCREATIC PROTEASES

Proteases are enzymes that digest proteins into smaller peptide fragments and amino acids by hydrolyzing the peptide bonds. Pancreatin contains five major proteases: trypsin, elastase, chymotrypsin, carboxypeptidase A1, and carboxypeptidase B. Trypsin, chymotrypsin, and elastase are classified as both serine proteases and endopeptidases because they cleave peptide bonds at the C-terminal side of an amino acid and also have a catalytically important serine residue in their active sites. Carboxypeptidases catalyze hydrolysis of the amino acids from the C-terminal end position in polypeptides and thus are classified as exopeptidases. Carboxypeptidases sequentially release residues from the C-terminus of proteins and peptides with a well-defined specificity.

Trypsin acts specifically on the C-terminal side of the positively charged amino acid residues lysine and arginine. Trypsinogen is secreted by the pancreas as an inactive precursor and discharged into the duodenum where enterokinase converts trypsinogen to active trypsin. Enterokinase is secreted in the duodenum by cells of the duodenal mucosa. Enterokinase removes a terminal octapeptide from trypsinogen and yields a polypeptide chain of active trypsin cross-linked by six disulfide bridges. Trypsin contains one high-affinity calcium binding site that is required for enzyme stability.

Chymotrypsin acts preferentially on the C-terminal side of tyrosine, phenylalanine, and tryptophan residues. It is secreted as an inactive zymogen, chymotrypsinogen, which undergoes proteolytic processing by trypsin to form the active enzyme. Chymotrypsin binds one calcium ion per molecule.

Elastase acts on small, neutral amino acid residues, such as glycine and alanine, but also hydrolyses amides and esters and is distinctive in that it acts upon elastin. Elastase is produced as a zymogen and the activated form contains four disulfide bridges. Elastase binds one calcium ion per molecule.

Carboxypeptidase A1 is an exopeptidase hydrolyzing the peptide bond adjacent to the C-terminal end of a polypeptide chain, thus releasing the C-terminal amino acid. It cleaves aromatic and bulky aliphatic amino acid residues and shows little or

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no action with aspartic acid, glutamic acid, arginine, lysine, and proline amino acid residues. It contains one zinc ion per molecule. The zinc ion is essential for activity; if removed during dialysis it must be replaced. Thus, carboxypeptidase A1 is also classified as a metalloprotease.

Carboxypeptidase B catalyzes the hydrolysis of the basic amino acids lysine, arginine, and ornithine from the C-terminal end of a polypeptide chain. It may also have a function in the further degradation of products of tryptic digestion. The enzyme binds one zinc ion per molecule, which is a necessary functional part of the enzyme for activity, and thus carboxypeptidase B is also classified as a metalloprotease.

7.2.2 PANCREATIC LIPASE AND COLIPASE

Pancreatic lipase (PL, also known as triacylglycerol acyl hydrolase) is a glycoprotein and produced directly as an active enzyme by the pancreas. Two glycosylation isoforms of PL, lipase A and lipase B, are present in pancreatin. These two isoforms have identical amino acid compositions but differ slightly in their glycosylation patterns, with lipase A as more acidic than lipase B. PL is a water-soluble enzyme that acts on insoluble lipid substrates, triglycerides, at the lipid–water interface. Its activity is dependent on the substrate-specific surface accessible to the enzyme, and it increases with the state of emulsification of the lipids. PL preferentially hydrolyzes ester bonds at the C-1 and C-3 positions of triglycerides and exhibits a broad spectrum of fatty acid chain length specificity. Therefore, PL is active against a wide variety of the triglycerides that are typically present in the diet. It also acts on diglycerides, but its activity on monoglycerides is very weak. It mainly converts triglycerides into monoglycerides and free fatty acids; the more polar lipolysis products are absorbed in the small intestine. In the presence of various amphiphiles such as bile salts at micellar concentrations, the PL adsorption at the lipid–water interface can be hindered, thus decreasing lipolytic activity.

A small protein cofactor also produced by the pancreas, colipase, helps PL to anchor to interfaces in the presence of competitive amphiphiles, and thus restores PL activity. Colipase is also produced by the pancreas as a precursor, procolipase. Procolipase is activated by trypsin.

7.2.3 PANCREATIC PHOSPHOLIPASE A2

Phospholipase A2 (also known as type IB secretory PLA2) is a heat-stable, water-soluble enzyme that catalyzes the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides. It is produced as a precursor that is activated by trypsin.

7.2.4 PANCREATIC AMYLASE

Porcine pancreatic α-amylase (1,4-α-D-glucan glucanohydrolase) catalyzes the hydrolysis of internal 1,4-α-D glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units to yield a mixture of dextrins, maltose, and glucose. Amylase exists in two forms (I and II) that have similar enzymatic properties but that differ in their isoelectric points. Both molecular forms of amylase are glycoproteins that contain fucose, galactose, mannose, and different contents of glucosamine. Both amylases consist of a single polypeptide chain with four disulfide bridges and contain a tightly bound calcium ion.

8. ENZYME ACTIVITY MEASUREMENTS

Although pancreatin contains a variety of enzymes, it is usually characterized by measuring the activities of the three main enzyme classes, lipase, protease, and amylase.

8.1 Lipase Activity

The lipase activity of pancreatin on triglycerides with long-chain fatty acids is mainly due to PL, which requires the presence of its specific cofactor, colipase, to act on triglyceride emulsions in the presence of bile salts that are competitors for lipase adsorption at the surface of lipid droplets. Colipase and lipase form an active complex with a 1-to-1 stoichiometry. Commercial preparations of pancreatin usually contain colipase; however, it is recommended to check that the API manufacturing process provides enough colipase for the lipase activity. This control should be part of a proper characterization to be performed during process validation of the API.

Besides PL, pancreatin also contains pancreatic carboxyl ester lipase (CEL), which hydrolyzes several lipid substrates. CEL activity on triglycerides is usually considered very low, compared with that of PL, and its contribution to pancreatin lipase activity is negligible, as measured by the USP lipase assay.

The lipase assay described in the USP *Pancreatin* monograph is based on the enzyme's rate of digestion of olive oil emulsified with acacia (also known as gum arabic). The activity of the test sample is calculated by comparing it to a standard preparation of enzyme with known activity. Olive oil contains triglycerides with long-chain fatty acids that are representative of dietary triglycerides. Lipase activity is measured on the basis of the titration of the free fatty acids released from olive oil upon lipolysis by the lipase present in pancreatin. This titration by sodium hydroxide is performed at a constant pH of 9.0 by pH- or potentiostatic titration, at which long-chain fatty acids are totally ionized. It is worth noting that this pH value does not correspond to physiological conditions (the mean pH of the small intestine contents is close to 6.0 during a meal), but it allows measurement of optimum lipase activity *in vitro*. Because the USP lipase assay solution contains USP Bile Salts Reference Standard (RS), the detection of enzyme activity requires that both lipase and colipase are present in pancreatin. One USP Unit of lipase is defined as the amount of enzyme that, under the defined conditions with the defined substrate, liberates 1 µmole of fatty acid per minute.

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Other lipase assays are available for monitoring lipase activity in pancreatin. When using lipase assays with non-natural substrates, such as tributyrin, it is recommended to confirm that the pancreatin sample tested is also active on long-chain triglycerides, particularly using the USP lipase assay with olive oil–acacia emulsion.

8.2 Protease Activity

The proteolytic activity of pancreatin on polypeptides and proteins is due to the inherent enzymes trypsin, chymotrypsin, elastase, carboxypeptidase A1, and carboxypeptidase B. The two major pancreatic proteases are trypsin and chymotrypsin, and along with elastase, these endopeptidases generate small polypeptides from larger proteins. The further action of the exopeptidases carboxypeptidase A1 and B leads to single amino acids during digestion.

The USP spectrophotometric assay for protease activity from pancreatin is based on the enzyme's rate of digestion of casein under test conditions. The activity of the test sample is calculated by comparing it to a standard preparation of enzyme with known activity. Casein is composed of α (s1) and α (s2)-caseins, β-casein, and κ-casein, which are phosphorylated on serine residues and lack disulfide bridges. The conformation of casein is similar to that of denatured globular proteins with little or no tertiary structure. It is recommended that users evaluate suppliers of the casein substrate for consistency of dispersion.

The hydrolysis of casein by pancreatic proteases generates single amino acids and small peptides, and their release can be quantified by measuring their absorption at 280 nm. Before this measurement, non-hydrolyzed proteins and large peptides have to be separated by a selective precipitation with trichloroacetic acid, followed by filtration. The filtrate is then used for the spectrophotometric assay of protease activity, using tyrosine as a calibrant. One USP Unit of protease activity is contained in the amount of pancreatin that hydrolyzes casein at an initial rate such that the amount of peptides liberated per minute and not precipitated by trichloroacetic acid gives the same absorbance at 280 nm as 15 nmol of tyrosine.

Other assays are available for monitoring the activity of individual and total proteases in pancreatin, and the unit assignments are specific for each substrate. *N*-Acetyl-L-tyrosine ethyl ester is commonly used for both titrimetric and spectrophotometric (λ = 237 nm) assays of chymotrypsin activity. Similarly, *N*-benzoyl-L-arginine ethyl ester is commonly used for both titrimetric and spectrophotometric (λ = 253 nm) assays of trypsin activity. In both cases, the titrimetric assay is based on ester hydrolysis by proteases and the release of acid groups, whereas the spectrophotometric assay is based on the chromogenic properties of these acids. Toluene-sulfonyl-L-arginine methyl ester is another chromogenic substrate (λ = 247 nm) used for measuring trypsin activity. These substrates are, however, not proteins, and the assays involve the cleavage of a carboxylic ester bond instead of a peptide bond.

Other assays using chromogenic peptides as substrates have been developed for improving specificity and sensitivity. More specific and sensitive protease assays have been developed using short synthetic peptide substrates (3–5 amino acid residues) with a chromogenic group (4-nitroaniline) coupled to the C-terminal end by an amide bond. The chromogenic group is specifically removed by proteases and is measured photometrically. The change in the absorbance at 405 nm is directly proportional to the protease activity. Specific substrates are commercially available for trypsin (carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate) and chymotrypsin (methyl-*O*-succinoyl-arginyl-prolyl-tyrosine-4-nitril-anilide chlorhydrate). Fluorescein isothiocyanate (FITC)-labeled casein is also used as a general protease substrate. The assay is based on the quenching of the fluorescein label bound to casein. When FITC-casein is digested into smaller peptides by proteases, fluorescence at 530 nm (excitation at 485 nm) is increased and can be measured to determine protease activity.

8.3 Amylase Activity

The glycolytic activity of pancreatin is due to α-type amylases. Such enzymes catalyze the hydrolysis on internal α-1,4-glucan links in polysaccharides containing three or more α-1,4-linked D-glucose units, yielding a mixture of maltose and glucose. The two major isoforms (I and II) of porcine amylases have identical enzymatic properties. In the last decades, several methods have been developed for assaying amylase activity; many of these are based on the detection of starch hydrolysis, as this polymer is the natural amylase substrate. Starch consists of two types of molecules, amylose (usually 20%–30%) and amylopectin (usually 70%–80%). Both polymers result from the assembly of glucose units connected via α-1,4-glucan links; in addition, in amylopectin, about 1 residue in every 20 or so, is also linked α-(1→6), forming branch-points.

The USP Amylase assay in the *Pancreatin* monograph is based on the enzyme's rate of digestion of starch, and the activity of the test sample is calculated by comparing it to a standard preparation of enzyme with known activity. Starch is hydrolyzed by amylase; the reducing groups resulting from the hydrolysis react with iodine in alkaline solution; and the excess iodine is titrated with thiosulphate. One USP Unit of amylase activity is defined as the amount of pancreatin that decomposes starch at an initial rate such that 0.16 µEq of glycosidic linkage is hydrolyzed per minute under the conditions of the assay.

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⟨1027⟩ FLOW CYTOMETRY

INTRODUCTION

Flow cytometry is an analytical method that plays a critical role in the quantitative and qualitative assessment of cell populations in patient and cellular product samples. The power of flow cytometry lies in its ability to rapidly and reliably analyze multiple attributes of individual cells within heterogeneous cell populations. Despite the value of flow cytometry data, method validation is challenging—perhaps more so than for other analytical methods—because of errors and artifacts from multiple sources.

Although flow cytometric methods can also be used to sort and isolate cells as part of the manufacturing process for cell- and tissue-based biological products, the scope of this chapter is limited to the use of flow cytometry as an analytical method. This chapter presents the technical aspects of the method, including instrumentation, sample handling and staining, and data analysis. Sources of error are considered in the context of technical features, as well as in the discussion of quality control, quality assurance, and standardization. Finally, current applications and assay troubleshooting principles are presented. For additional information on the basics and practical aspects of flow cytometry, see the current edition of *Practical Flow Cytometry* (Shapiro, 2003).

Flow cytometry is widely used to characterize cell and tissue-based products, but most assay methods are not yet standardized. In addition to issues related to technical complexity, there are also challenges to standardization of flow cytometric

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methods for specific product classes or types related to the heterogeneous nature of these products, even among those with similar manufacturing processes and clinical uses. Current and future innovations in instrumentation, analytic reagents, analytic algorithms, and automation are likely to improve the technology's capabilities but are unlikely to eliminate challenges (e.g., bioassay, identification tests, and other applications).

PRINCIPLES OF OPERATION, METHODS, QUALITY, AND STANDARDIZATION

The process of flow cytometry requires that cells move past a fixed light source consisting of one or more lasers so that individual cells can be observed or interrogated for characteristics such as size, granularity, and presence of surface membrane or intracellular antigens or molecules. The cells are suspended in fluid in which movement is controlled by the size and configuration of tubing, chambers, and pumps specific to the flow cytometry instrument. The pattern of light signals produced from the laser light's interaction with the cells is captured by a detection system, also specific to the instrument, and the detected signals are transformed into data elements that can be analyzed and combined with data from other cells in a given sample. Data from a cell suspension can then be expressed and presented in one-, two-, or three-dimensional visual formats, or in numerical formats, to characterize the cellular sample and its subpopulations both qualitatively and quantitatively.

Flow Cytometry Instrumentation

Flow cytometers, which incorporate fluidic, optical, and electronic signal processing elements, are described below.

FLUIDICS

The fluidics system moves a bulk mixture of cells so that a stream of single cells is formed. Within the flow cytometer, the single-cell suspension passes through a confined region where each cell is sequentially illuminated by a uniform light source at the observation point (interrogation point). Most instruments use a flow chamber (flow cell) that, after the cell sample is drawn into the sample injection tip, combines the cells with isotonic sheath fluid, using a conical nozzle assembly that is geometrically designed to produce a laminar flow of fluid (*Figure 1*). The fluid outlet nozzle typically has an orifice of 50–250 µm in diameter through which fluid exits at a high flow rate. Differential pressures between the sample stream of cells (lower pressure) and the sheath stream (higher pressure) draw the cells/particles out into a confined stream. The resulting coaxial stream within a stream is highly efficient, and the sample stream at the observation point is typically only slightly larger than the cells or particles contained within. At least one manufacturer uses an alternative approach in which the coaxial stream strategy is replaced by the use of microcapillaries to focus and direct the cells. The light signal deflected or emitted by the cell is then measured and analyzed.

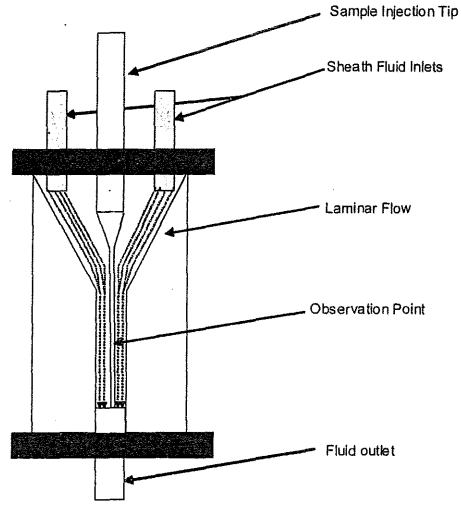

Figure 1. Schematic diagram of a flow cell.

OPTICS

When cells are stained with fluorescent dyes or with fluorescent-labeled antibody reagents, light emitted from the laser interacts with the fluorescent dye to produce a stimulated emission that has coherent (parallel) waves of light of uniform wavelength, phase, and polarization. Fluorescent light signals generated from the interaction of the laser light with the cells are collected by an array of detectors oriented in direct line with, and at 90° to, the incoming laser beam. The most common commercially available flow cytometer lasers (with corresponding wavelengths) are the blue argon laser (488 nm), the red diode laser (635 nm), and the violet laser (405 nm).

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ELECTRONIC SIGNAL PROCESSING AND DATA OUTPUT

When a cell passes through the optical system of a flow cytometer, the light-scattering patterns or fluorescence from any fluorochrome on or in the cell are detected by various types of photodetectors or photomultiplier tubes (PMT) that transform the information about the characteristics of the cell into a computerized readout. Each analyzed cell generates an event in each parameter (forward scatter, side scatter, fluorescence) for which it is measured. *Figure 2* shows an example of a typical two-color flow cytometer configuration. Different cell types have distinctive sets of signals in the various parameters. For example, when the cell passes through a beam of light, the light deflected in the forward direction (usually about 20° from the forward direction of the laser) is called forward scatter and is collected by a detector known as the forward scatter channel (FSC). The amount of deflection in the FSC is proportional to cell size. Light deflected at a 90° angle is known as side scatter and is collected by the side scatter channel (SSC). This provides a measure of the cell's structural complexity caused by granules, membrane roughness, or nucleus, all of which are associated with higher SSC. The light deflected by other PMTs using a specific band-pass filter is collected by specific fluorescence channels (FL1 and FL2 in *Figure 2*). The electrical pulses, originating from light detected by the PMTs, are processed by a series of linear and log amplifiers. Logarithmic amplification is often used to measure fluorescence in cells. *Figures 3–7* show histograms for cells stained with antibodies conjugated with specific fluorochromes (see *Table 1*). The antibodies are specific to some of the cluster of differentiation (CD) markers discussed in *Immunophenotyping* (see below).

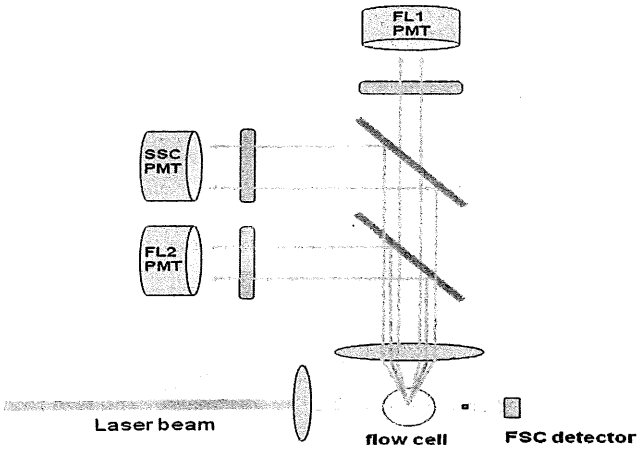

Figure 2. Typical 2-color flow cytometer with detectors for FSC, SCC, and fluorescence.

Table 1. Fluorochromes Commonly Used in Flow Cytometry

Fluorochrome	Typical Excitation Laser (nm)	Emission Peak (nm)
Cascade Blue	375; 401	423
Pacific Blue	403	455
R-Phycoerythrin (R-PE)	480; 565	578
PE-Cy5 conjugates	480; 565; 650	670
PE-Cy7 conjugates	480; 565; 743	767
Red 613 (Texas Red)	480; 565	613
Peridinin Chlorophyll (PerCP)	490	675
Fluorescein (FITC)	495	519
Allophycocyanin (APC)	650	660
APC-Cy7 conjugates	650; 755	767

The versatility of flow cytometry comes from the ability to attach fluorescent tags to the cell's surface, cytoplasm, or nucleus or to products of the cell. Fluorescent markers attached to the cell can be excited by lasers to emit light of specific wavelengths, and this light is then detected and analyzed in the manner described above. The type and amount of fluorescence detected provide both quantitative and qualitative information about the cell.

The photodetectors convert light into an analyzable output by generating a small current of which the voltage has amplitude proportional to the amount of light. The voltage is amplified and converted into electrical signals large enough to be plotted by the computer in several different ways. Thus, the FSC, SSC, and fluorescent detectors collect the light and convert it into

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electrical signals that can be analyzed by the computer. In this way, the signals coming from each photodetector can be measured for their intensity (low to high) and sorted into channels. The channels are arranged as a continuum so that a cell population with many large cells will have many events in the higher channels, and one with many small cells will have many events in the lower channels.

DATA ANALYSIS

Data output from the flow cytometer can be represented in several ways, the most basic of which is the single-parameter histogram (*Figure 3*), in which events with similar intensity of light (forward scatter, side scatter, or fluorescence) are collected in channels and then plotted. This plot demonstrates the number of cells with similar optical characteristics. *Figure 4* is an example of graphs that display two measurement parameters, one on the *x*-axis and one on the *y*-axis, and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC, or fluorescence. These parameters can be collected in one channel.

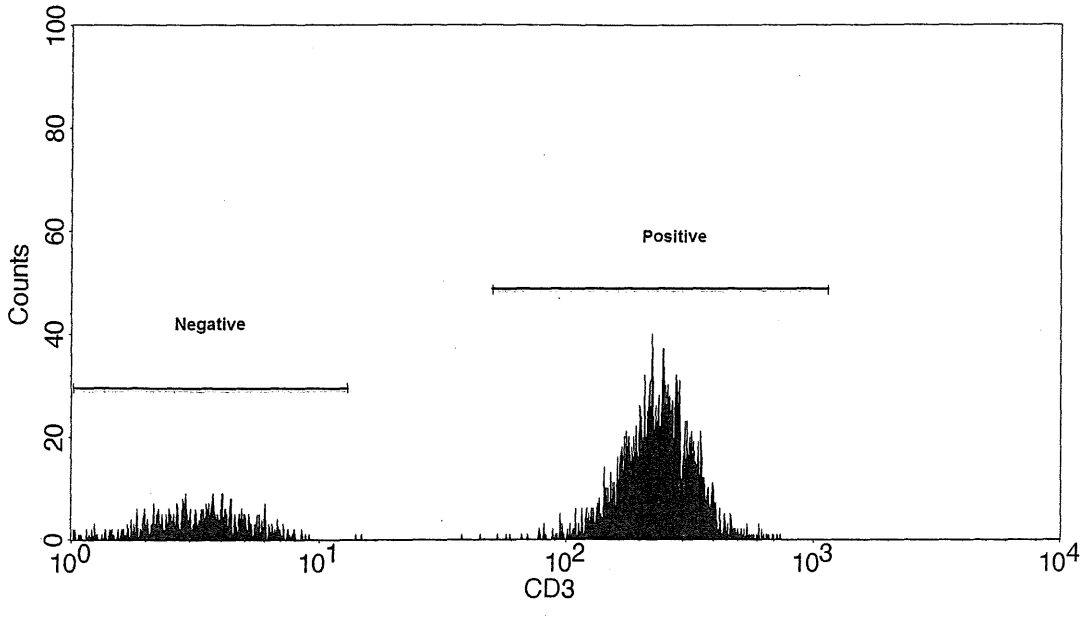

Figure 3. Single-parameter histogram showing expression of the cellular antigen CD3 in a mixture of cells.

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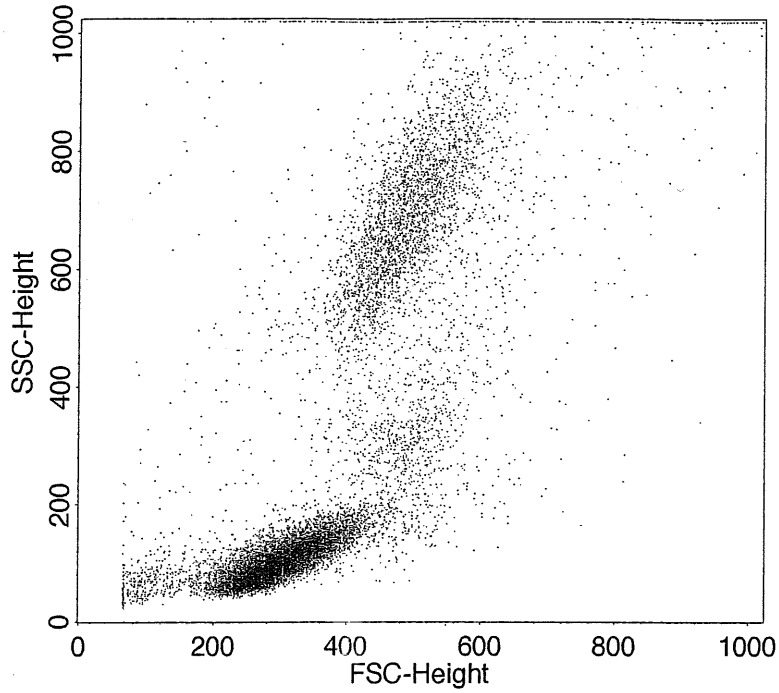

Figure 4. Bivariate dot plot of cells displayed by FSC and SSC.

A dot plot displays a dot for each cell, and density plots and contour plots show a heat map or a topographical linear map, respectively, based on the relative number of cells in each channel. The forward versus side scatter histogram is the most common method of identifying different hematopoietic cell types. *Figure 5* shows a contour plot that is a 3-dimensional representation of the relative number of cells in the various channels.

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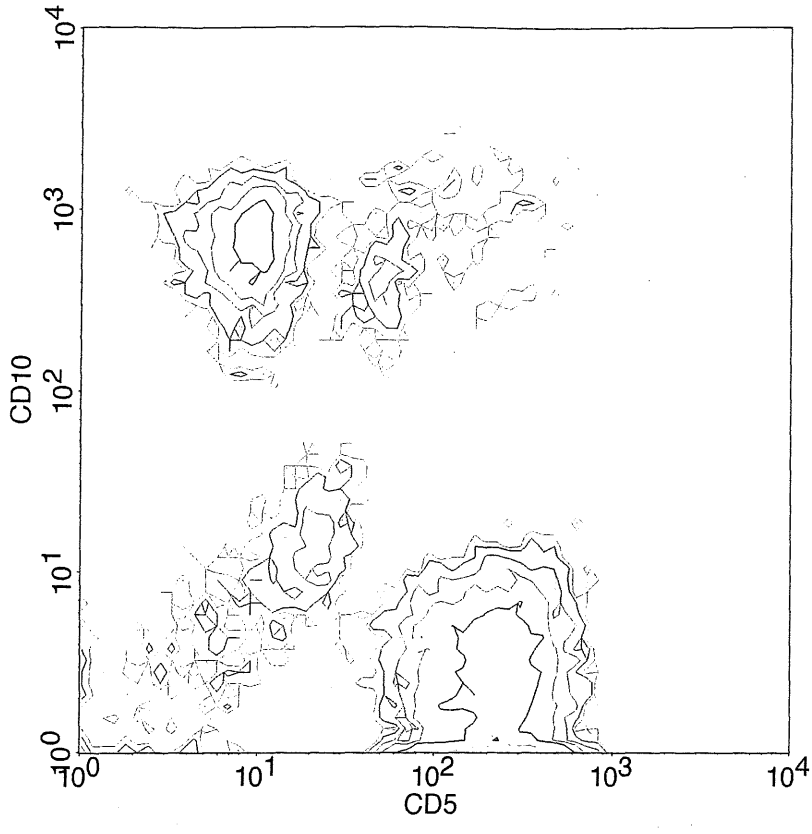

Figure 5. Bivariate contour plot showing relative numbers of cells present in each channel that co-express 2 CD markers.

When cells are stained with antibodies for different epitopes carrying two different fluorochromes, the data are presented as a plot of the two parameters plotted against each other. Cursors can be set on each axis to separate positive populations from negative populations for each of the attributes. This results in a graphic representation of cells that are positive for both markers, negative for both markers, and positive for only one of the two markers (*Figure 6*).

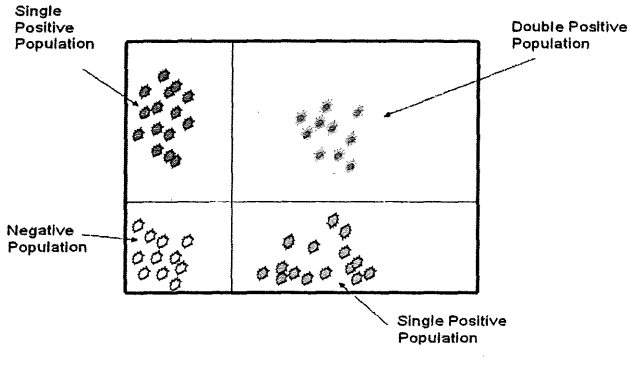

Figure 6. Schematic presentation of a 2-parameter histogram.

The flow cytometer allows the user to set the limits of positive and negative for each marker. Flow cytometric data are collected in list mode, where each electronic signal from a respective cell is displayed in the sequence in which it was acquired. List mode files can be edited at a later time to include or exclude any event. A basic advantage of flow cytometry is the ability to separate the data about the cells of interest from both the background and dead cells (e.g., noncellular particles or debris) when dealing with forward and side scatter and cells of other populations. The user must decide which signals are the actual light outputs from the cells and must construct an electronic gate to tell the computer to count as positive only the events that fall within the gate. Cell populations can vary widely depending on the tissue or cell source and the characteristics of the flow cytometer used. Gating allows the user to determine which outputs to consider actual events, so this process is of prime importance in standardizing flow cytometry data (*Figure 7*).

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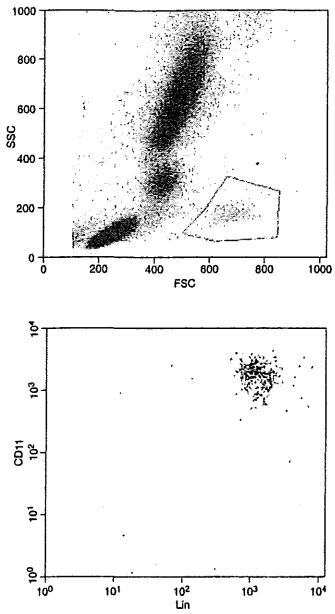

Figure 7. Gating of a cell population with low side scatter and high forward scatter, which is distinct from other cell populations in the sample.

A technique known as compensation can be used to separate spectral overlap of fluorochromes that have similar emission wavelengths. For analog-style flow cytometers manufactured before the late 1990s, compensation must be set before data acquisition. In modern digital instruments, compensation can be set either before or after data acquisition. The adjustment of compensation can be more of an art than a science, and considerable literature has been focused on the relative merits of various methods to determine the correct compensation for cell types or experimental conditions. The analyst should have considerable knowledge of the cell type under analysis in order to prevent errors in phenotyping that can result from improper adjustment of compensation.

The number of events counted should be adequate for statistical confidence in the results. The instrument can be set so that data are collected until a certain number of events in a channel have been measured. This feature allows the operator to vary the length of time or number of events from the sample so that statistically reliable data are generated. Thus, a sample that measures a rare event will analyze more total cells than one that measures a common event. Use of list mode files, the electronic data files that represent the most uncensored data, provides an advantage because these files can be further analyzed after data acquisition. Investigators need to ensure that all raw data, documentation, protocols, specimens, and final reports are archived at the close of the study. To ensure the integrity and quality of raw data collected, researchers need to abide by U.S. FDA Regulations for Good Laboratory Practices (GLP) as prescribed in 21 CFR Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies; and Part 11, Electronic Records and Electronic Signatures.

Flow Cytometry: Elements of a Procedure

Flow cytometric methods incorporate sample handling, preparation, and staining; instrument setup and operation; data collection, analysis, and storage; and associated quality control measures.

SAMPLE HANDLING AND STAINING

Sample Collection, Handling, and Anticoagulation

In order to make accurate conclusions about the cell-based drug product, the analyst should ensure that samples from cellular products are as representative as possible of the whole product. Blood, apheresis samples, and cell suspension products should be well mixed before sampling, and care should be taken to obtain adequate sample volumes.

Cellular samples containing human blood/plasma must be anticoagulated. Citrate-based anticoagulants (e.g., Anticoagulant Citrate Dextrose Solution A) or heparin are recommended more highly than EDTA because they will optimally preserve samples being held for more than a few hours. For longer-term samples, specific transport/storage media may be required, and validation studies should be performed to ensure that those samples are equivalent to fresh samples at the time of flow cytometric analysis.

Samples intended for whole blood lysis and surface antigen staining should be transported and stored, preferably on a slow oscillating mixer, at room temperature. Fixed samples or live cell preparations should be stored at 4°. When the sample may be exposed to extreme temperatures, temperature-control materials (room temperature packs, wet ice/cold packs, and insulation) may be necessary, and validation studies should be performed to ensure sample integrity. For critical or high-value samples, temperature-monitoring devices may be needed during transport.

After acquisition, specimens should be analyzed as soon as possible. Special attention should be given to situations in which cellular proliferation and metabolic depletion of energy sources within the transport/storage media can lead to apoptosis. When accurate counting is not required or if infectious agents are suspected, a commercial lyse/fix solution can increase storage time

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and reduce the risk of disease transmission. For specimens separated by density-gradient centrifugation, storage in a solution of buffered paraformaldehyde (0.1% to 2.0%) is recommended after cell labeling has occurred.

Sample Processing, Staining, and Fixation

Reagents used in sample processing, staining, and fixation should be qualified for their intended use. Further guidance is available in ICH Q6B, *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*. When using reagent kits, follow the manufacturer's sample processing instructions.

Sample processing that involves centrifugation, washing, red blood cell (RBC) removal or lysis, or density-gradient separation is commonly done during many flow cytometric applications but can introduce error and artifact. Several techniques and reagents are available for RBC removal and lysis. Clinical grade [in vitro diagnostics (IVD) or analyte-specific reagents (ASR)] reagents are recommended for optimal quality, but artifacts can still occur. Density-gradient centrifugation can introduce error associated with variable cell losses among subpopulations that are being measured. These sources of error and artifact can be avoided by analyzing live whole blood whenever possible.

Most whole blood lysate instructions recommend staining at room temperature and in the dark. Many methods include a dilute fixative to prevent capping and internalization of fluorochrome. In contrast, cell preparations (density-gradient cell preparations, apheresis specimens, tissue culture) should be stained at 4°, washed with cold buffer, and stored cold until analyzed.

Fixation that also preserves cell surface antigens can be accomplished using commercial leukocyte preservatives or with buffered formaldehyde or paraformaldehyde. Very little validation of storage times, antibody binding, or fluorochrome intensity has been reported. Any laboratory that considers batch analysis of fixed specimens should validate these techniques thoroughly before implementing.

USE AND CHOICE OF FLUOROCHROMES

Fluorochromes

Fluorochromes are used for direct staining of cells or as agents bound to antibody or other reagents to stain cellular antigens or other structures. *Table 1* lists examples of common fluorochromes used for flow cytometry and their excitation and emission wavelengths. Wavelengths (nm) may vary slightly depending on the environment. Synthetic probes from specific manufacturers are also available.[1] Fluorochromes must match the spectral range for the lasers and filter sets specific to the user's flow cytometer.

When choosing fluorochromes for multicolor phenotyping, the operator should refer to established methods for the particular instrument. In general, the brightest fluorochromes should be matched with the antigens that are expected to have the lowest expression on the cell surface. The brightness of tandem dyes can also be reduced by the use of certain fixatives, some of which are less problematic than others. When designing a multicolor flow and tandem dye procedure that has not been previously validated, the operator should consult the manufacturer's technical service, compare tandem dye/fixative combinations, and validate the final fluorochrome combination to ensure sample-to-sample consistency.

Tandem fluorescent dyes are dual-conjugated fluorescent molecules. When the two labels are in close proximity, energy produced by the laser exciting the donor fluor is transferred to the acceptor fluor, releasing a photon at the emission wavelength of the acceptor fluor (also known as fluorescence resonance energy transfer, or FRET). For example, PECy5 will excite at the excitation wavelength for PE (565 nm), transfer energy to Cy5, and emit at the emission wavelength for Cy5 (670 nm).

Fluorescently Labeled Antibodies

Most commercially available antibody reagents are monoclonal, but polyclonal reagents may be available, and desirable, for some applications. The quality and specificity of an antibody can vary widely. Antibodies directed at a given antigen may differ in their binding specificity for different antigenic epitopes or in the strength of binding to the same epitope. If possible, use directly conjugated fluorochrome–antibody combinations that are IVD or ASR grade. Optimization of antibody concentration for the desired cell population is protocol specific but is generally accomplished by using increasing concentrations of antibody with a fixed number of cells to bracket the optimal brightness between autofluorescence and quenching. Quenching is caused by the prozone phenomenon, which occurs when excess antibody leads to immunoprecipitation and loss of fluorescence intensity. Further details are available in methods manuals such as *Current Protocols in Immunology* (Coligan et al. 1994).

Cell Surface Antigen Staining

Techniques for surface antigen staining vary with the type of specimen. Whole blood lysis techniques generally require surface labeling at room temperature in darkness for 15–30 min, followed by RBC lysis and, if desired, fixation. Published techniques use ammonium chloride (NH_4Cl) lysis of whole blood or marrow specimens followed by washing before antibody labeling for leukemia immunophenotyping. Mononuclear cell or cultured cell samples that are stained live should be kept at 4° or in an azide-containing buffer to prevent capping and internalization of the antibodies.

Intracellular Staining

Several standardized procedures also exist for labeling intracellular antigens and cytokines. The operator should consult the manufacturer's protocol and standardized reagents for these procedures. Because permeabilizing reagents vary among procedures and manufacturers, do not mix and match reagents. For cytokine labeling it is often necessary to use an activating step and a Golgi block to allow a sufficient amount of cytokine to accumulate for detection. If standardized reagents or procedures are not available from the manufacturer or if analysis of specialized functions is required, many common procedures and techniques can be found in sources such as *Current Protocols in Immunology* (Coligan et al., 1994).

Quantitation of Antigens

Some applications require quantitation of the average number and density of antigen molecules per cell in order to give a more complete picture of the immunological behavior of cells (e.g., in studies where extracellular antigens are expressed differentially in relation to activating stimuli). The intensity of an antibody/fluorochrome labeled cell preparation is compared to the intensity of a set of microbead fluorescence standards collected at the same PMT voltage settings. The standards are

[1] AlexaFluor series (InVitrogen/Molecular Probes) or the Cy series (GE Healthcare).

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calibrated in molecules of soluble fluorescence (MESF) units, from which one can determine effective fluorescence to antibody (F/P) ratio, the number of antigen molecules per cell, and the density of available sites per cell.

INSTRUMENT SETUP AND OPERATION

Compensation

Most instrument manufacturers supply software and test reagents (usually fluorescent beads) to set PMTs and compensation in order to target values found with the most common clinical tests (e.g., lymphocyte phenotyping, CD34 analysis). The operator should also use a biological control such as preserved blood or mononuclear cells, which are commercially available. Compensation must be set before acquisition on analog instruments. Digital instruments' PMTs must be set correctly because the values for these settings cannot be changed once the list mode file has been generated. When rare events are examined and/or intracellular dyes (e.g., 7-amino actinomycin D [7-AAD], propidium iodide [PI], Syto-16, etc.) are used in conjunction with fluorescently labeled antibodies, the balance of PMT voltage and spectral overlap must be closely monitored.

Autofluorescence (AF) is fluorescence above baseline in the absence of fluorochrome staining. This occurs in some cells, typically myeloid cells (especially alveolar macrophages) and cultured primary cells. If desired or necessary, AF can be measured directly on a fixed PMT voltage or can be calculated from a reference standard of fluorescent reference bead preparations (see *Quantitation of Antigens*, above). Avoid use of the 488 or 532 nm excitation wavelength and subsequent spectral compensation of the AF as an additional fluorochrome.

Data Acquisition and Gating Strategies

When possible, all events should be acquired in list mode, i.e., without selective gating of events. *Live gating*, defined as selective gating of events during acquisition, should be employed only when the desired subset is sufficiently rare that >2 million total events must be analyzed in order to count a significant (100 or greater) number of events of the desired population. List mode data can be acquired uncompensated when digital instrumentation is used, but most operators find that analysis is much less difficult and time-consuming if the data are in the range of proper compensation before acquisition. In addition, it is often desirable to set thresholds for exclusion of debris. Setting a forward scatter threshold, for instance, excludes events below a predetermined size in order to prevent the large list mode file size that can occur when these events are counted.

Use of Controls

Fluorochrome-conjugated bead preparations are used for standardizing PMTs and compensation and for quantifying the expression of specific markers. The use of biologic controls is also highly recommended. Stain the cell samples with an isotype control and primary and secondary antibodies to assess nonspecific binding unless the laboratory has ascertained by rigorous validation procedures that nonspecific binding does not interfere with assay results.

Antigen-positive and -negative cell populations (prepared and stained in a manner identical to that for the test articles) provide internal system suitability standards. Such control cell populations also allow the laboratory to assess lot-to-lot variations in antibody preparations and staining reagents.

Use of Dyes and Gating for Cell Viability

Cell viability dyes such as 7-AAD, PI, and TO-PRO iodide are commonly used to determine the proportion of dead cells in a cell therapy product. These dyes are typically excluded from live cells but pass through the cell membranes of dead cells, staining their DNA. Cell viability staining can be combined with surface membrane or intracellular staining to evaluate subpopulations and the proportion of live and dead cells stained with a given marker. Viability staining can also be used in conjunction with a membrane dye in flow cytometry-based cytotoxicity assays. These viability dyes should not be confused with the many apoptosis-detection reagents now available. Validation techniques for non-IVD viability dyes involve the preparation of a dead cell population that is added in serial dilution to a live cell product, and the cell mixture is then assessed for fidelity to the known proportion by staining with the dye of interest.

Cell Enumeration

Absolute cell count, expressed as the number of cells in a given sample volume, can be determined by dual- and single-platform methods. The dual-platform method relies on a separate automated cell-counting instrument or manual counting method to first enumerate the cell population. The percentage of a subset(s) of interest is then determined by flow cytometry, that percentage is multiplied by the cell count, and the result is divided by 100. Single-platform methods enumerate the cell population and subset counts directly by counting the cells in the sample simultaneously while counting reference beads that have been added to that sample volume in a known concentration. Reference beads are often provided as a bead suspension that is added to the specimen. Alternatively, a given volume of sample may be added to a known number of reference beads provided as a solid phase matrix in polystyrene tubes. These approaches are subject to pipetting error, so extra care must be taken to ensure accuracy and reproducibility.

Instrument Setup and Quality Assurance

Each laboratory should have a quality plan that defines the standard operating procedures for instrument setup and calibration, as well as regular instrument monitoring, maintenance, and cleaning. Instrument logs should document these activities and operators. In general, the instrument manufacturer's quality program should be followed unless a suitable alternative has been established.

Instrument parameters such as laser current, voltage, output, and PMT voltages during calibration should be monitored and recorded whenever the instrument is in use. Careful monitoring of instrument setup parameters can be helpful in detecting trends and predicting laser or PMT failure. Biological control testing results should also be monitored and recorded to detect and prevent analytical method drift.

The laboratory should also participate in a proficiency-testing program that reflects the test menu. Depending on need, this could range from a formal program such as the one administered by the College of American Pathologists (CAP) to simple sharing of specimens and analysis with another laboratory. Ensuring that operators are trained, qualified, and periodically evaluated for proficiency to perform specific procedures will also help ensure the consistency of techniques and controls.

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DATA MANAGEMENT AND STATISTICAL CONSIDERATIONS

Data Management and Storage

Quality control assays and sample test assays (in list mode) should be stored in a manner that complies with regulatory requirements applicable to the laboratory. This can be accomplished by transfer to fixed drives, removable media, or to a server such as a commercial laboratory information system. Storage of results should always be traceable to the original FCS list mode file, instrument settings, and quality control parameters for that particular specimen. Data should be backed up to avoid loss of files. Storage and backup procedures should also be established for manual (paper) records that may be used for calculations and summary data.

Data Analysis and Statistical Considerations

For most flow cytometry applications, data analysis involves displaying the data from list mode files or live gating in a plot (single-parameter histogram plot, two-parameter dot plot with regions, or three-dimensional plot), and measuring the distribution of events within that plot. Further analysis of data within selected populations can be done by gating on specific cell populations. Description of the data typically includes the percentage of events within the population with a given characteristic (forward scatter, side scatter, fluorescent marker). The numerator is the number of events with the characteristic, and the denominator is either the number of total events counted or the number of gated events counted. For two-dimensional plots, analysis is typically done using computer software that analyzes and reports regional (e.g., quadrant) statistics. Because cell population clusters may shift their positions from one data file to the next, software has been developed for cluster analysis.

Statistical analysis of quantitative flow cytometry applications differs from qualitative applications in which a cell is considered either positive or negative for a given marker. For a typical quantitative application in which the number of molecules on the cell surface is estimated, the mean or median fluorescence intensity of the sample cells labeled with a fluorescent antibody bound to the molecule of interest can be compared to appropriate controls, including standard curves of cells or particles with known quantities of that molecule/antibody.

A common practical consideration for flow cytometric analysis of cell therapy products, especially autologous and related donor allogeneic products, is that sample size for analytical testing is often limited because of the limited cell content of the therapeutic product itself. This creates special challenges if cells containing the flow marker of interest are rare events. In these cases, before making a decision about sample size, the user must consider the expectations for detecting the rare events within a given number of total cells (i.e., prevalence, variability, sampling error) in relationship to the desired precision of the estimate.

Quality and Standardization

Standardization of flow cytometry practices and equipment requires validation, quality assurance (QA), and quality control (QC) practices. Although flow cytometry is used widely in both research and clinical laboratories, testing of cells for the development of clinical diagnostic and therapeutic applications is increasing, leading to more comprehensive regulatory requirements and attention to standardization. As an example, flow cytometry operators have traditionally used fluorescent microspheres (beads) or cells for instrument setup and QC, frequently based upon manufacturers' recommendations. However, consistent instructions about how these control standards should be applied to instrument setup and QC remain elusive.

Properly applied, validation provides documented evidence that the manufacturing or testing process consistently produces product that meets predetermined specifications. Based on a thorough understanding of critical process parameters, validation helps to define product quality and helps to ensure a consistent and well-controlled manufacturing or testing process. Validation of flow cytometric methods should incorporate instrument qualification, analytical method validation, and operator qualification.

DOCUMENTATION

GLP and GMP processes require appropriate documentation such as standard operating procedures (SOPs) for all lab processes. SOPs must also be periodically updated and approved to reflect current practices. Training and qualification are required so that laboratory staff have the appropriate level of competence for their assigned responsibility. Operator competencies must be continually reviewed and assessed in relationship to SOPs and policies.

Integrating both internal and external quality processes is an important element of quality assurance. These involve equipment validation, manufacturing controls and limits, and product specifications. Process and equipment validation processes generally require installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

EQUIPMENT AND ASSAY QUALIFICATION

IQ establishes that the instrument is received as designed and specified, and that it is properly installed in a suitable environment. This generally means checking physical and facility requirements to determine whether the flow cytometer can be suitably installed. Qualification factors verified typically include temperature, humidity, space, and electrical facility capabilities in relationship to the instrument manufacturer's requirements. IQ procedures also ensure that all hardware and software components purchased are installed properly by the instrument manufacturer's representative.

OQ demonstrates that an instrument will function according to the manufacturer's specifications. This generally means component-level testing by the instrument manufacturer's representative or using a manufacturer's validation package that guides the end user to perform this function. Where possible, these tests should have specifications with corresponding quantitative control limits. This testing ensures that the instrument hardware and software are operational by comparison with the manufacturer's specifications.

PQ demonstrates that both the instrument and the assay consistently perform according to specifications. For flow cytometry, these specifications are generally determined by the laboratory performing the flow cytometric testing and usually include daily instrument and assay control test specifications. For specific assays, PQ should incorporate standardized methodology, application-specific setup and compensation, and specifications for linearity, precision, and accuracy of reported assay results.

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On some digital flow cytometers, a baseline instrument setup may be necessary in order to determine the optimal instrument settings for a given assay.

INSTRUMENT PERFORMANCE

Performance specifications may be identified by the flow cytometer manufacturer and may not address all of the operator's specification requirements. After identifying the manufacturer's specifications, the laboratory must establish specifications that are appropriate for the hardware configuration(s) that will be used, and the specifications must be standardized. As an example, the manufacturer may have a base specification for a four-color dual-laser flow cytometer. If the base specification calls for the standard red diode 635-nm laser but a helium–neon air-cooled 633-nm laser is substituted, the base specifications are no longer valid for the system. Similarly, if the specifications are based on the use of a 660-nm band-pass filter but a 675-nm long-pass filter is substituted, the base specifications are not valid because of differences in the emission filter characteristics.

PERFORMANCE MONITORING

Instrument performance can be monitored on a daily basis, using commercially available fluorescent beads. Light detectors such as PMTs, photodiodes (PD), and avalanche photodiodes (APDs) are used in most systems to detect signals, and their gains can be changed to increase or decrease the sensitivity of the detectors. Therefore, monitoring the settings of these detectors is as important as monitoring the signals, and these settings should be associated with each raw data file. The easiest approach is to maintain the same instrument detector settings from day to day and to measure the intensity of the fluorescence signals. This approach should be implemented for all parameters that must be validated using the appropriate beads. Instrument sensitivity is based on the ability of the detection system to resolve dim cell or bead populations. For this reason, measuring the coefficient of variation (CV) of dim to moderately intense fluorescent bead populations is a means to monitor fluorescence sensitivity on a daily basis. The instrument manufacturer's recommendations should be used to monitor performance.

Ambient high temperature can affect laser and PMT performance and should also be monitored on a daily basis.

Incorrect compensation for spectral overlap can strongly affect data during multicolor analysis. Many approaches have been established for this purpose, and recently mathematical algorithms have been used rather than analog circuitry. Algorithms, such as those that use matrix algebra, enable the operator or investigators to apply objective criteria to compensate for spectral overlap after all data have been collected. On older systems, the standard approach has been to compensate using a subtractive hardware adjustment to the observed preliminary data before all data have been collected. This approach can be subjective and is not as likely to produce accurate results as compensation by newer methods. Antibody-bound capture beads are valuable compensation tools because they can be used with the same antibody and tandem dyes for all fluorophores that will be used on cells. Validate the use of beads in place of cells for compensation purposes.

STANDARDS

Microsphere-based fluorescence standards for flow cytometry have been categorized by their purpose:

- Type I standards are alignment standards that are used to make adjustments to the instrument's optical alignment. These are typically used by field service engineers and by users of operator-adjusted systems to check the optical signal alignment in order to improve instrument sensitivity. These particles are typically small (≈ 2 µm) and bright, and they provide the most uniform illumination.
- Type II standards are reference beads and are the most commonly used bead standards. These typically are used on a daily basis, have dim to moderate fluorescence intensity, and can be obtained with various attached fluorophores. These can be used to mimic cells and, with dedicated software, to determine relative instrument sensitivity.
- Type III standards are used for fluorescence calibration. These are used for specialized applications that require calibration of one or more fluorescence detectors for quantitation of molecules of fluorochrome. Determination of the ratio of fluorophores to antibody (F/P ratio) allows subsequent calculation of the number of antibodies bound per cell.

INSTRUMENT SETUP AND QUALITY ASSURANCE

Instrument setup and quality assurance should be activities independent of the biological assay. Many variables related to instrumentation can lead to artifacts in the biological assay results. Two activities can be used for instrument quality assurance: baseline setup and daily setup.

Baseline Setup

On newer digital instruments it is desirable to establish a baseline setup of instrument settings that provide optimal sensitivity. This setup is not a daily procedure but should be performed if the instrument configuration is changed or if the instrument is serviced. Because PMT voltages and instrument configuration can strongly affect instrument sensitivity, this method should be used to provide objectivity as well as improved sensitivity. PMT voltages can be increased to a range that provides a lower CV (*Figure 8*). These settings can be, but may not always be, used for the biological assay.

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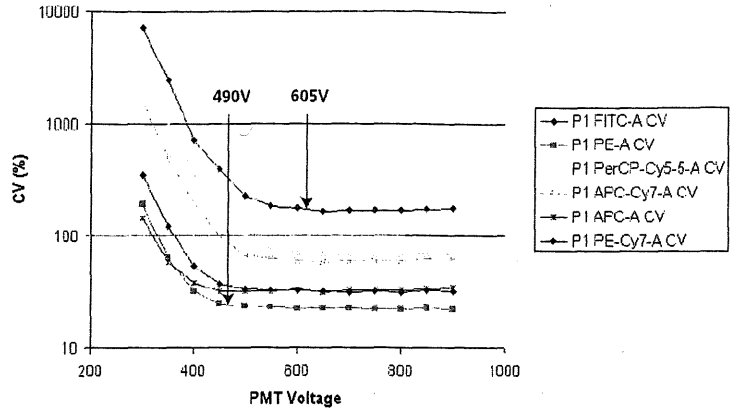

Figure 8. PMT voltages can be increased to a range that provides a lower CV.

Daily Setup

Type II standards, as reference particles, can be used to monitor signal intensity, separation of moderately bright and dim particles, and signal resolution. Fluorophore-matched beads provide a compensation tool as well as a means of knowing that the instrument is able to detect those wavelengths. Fluorophore-matched beads, however, do not have the fluorescence uniformity required for measuring CVs. Based on the optical performance of the instrument, CVs are best measured using dim and moderately bright hard-dyed beads such as coumarin-dyed microparticles that fluoresce in a broad spectral range.

It is easy to confuse assay controls and instrument controls. Beads provide a fluorescence uniformity and consistency that cannot be obtained with cells, and, accordingly, beads are useful for monitoring instrument performance. For this reason, it is better to use a process control cell preparation to verify compensation and fluorophore acceptability.

Instrument settings for daily setup are generally the same settings used for assays. Not all assays can be used with the same instrument settings, and it is not always necessary to perform these activities for every instrument setting unless it is required by validation.

Daily activities include consistent instrument setting from day to day, use of a broad range of dim to moderate intensity beads, and monitoring key parameters, including bead fluorescence intensity (absolute and % CV), PMT values, temperature, laser power, and laser wavelength.

ASSAY QUALITY CONTROL AND QUALITY ASSURANCE

Assay-specific instrument settings should be established to demonstrate that all cell populations can be identified in bivariate plots for fluorescence and light scatter. Most importantly, positive populations must be on scale and properly compensated. This is critical when exciting cells with red lasers, which do not cause cells to generate significant autofluorescence. For this reason, it is important to verify appropriate PMT settings so that the positive population is in the upper part of its fluorescence scale, because it may be extremely difficult to identify the negative cells.

Fluorescence compensation is a critical adjustment. Digital instruments provide objective offline adjustments during analysis, and detailed instructions for proper compensation settings are available. Using cells or capture beads stained with a single antibody-fluorochrome is generally the best approach, but specialized fluorochrome-labeled bead mixtures can also work well to compensate for multicolor acquisition and analysis.

ISOTYPE CONTROLS

An isotype control is a negative-control antibody that should not react with the antigen of interest and is the same isotype as the test antibody. Myeloma protein or immunoglobulin that has no specificity to the species being tested and has the same Ig chain class and subclass as the test antibody is conjugated to a fluorochrome identical to that on the test antibody. Ideally, very little or no binding occurs when the isotype control is used in parallel with the test. Idiotypic nonspecific binding frequently occurs, however, and is independent of the isotype of the antibody. This is most likely related to other differences in antibody chemistry and can be especially problematic with rare-event detection assays, such as those for hematopoietic stem cell assays in peripheral blood.

FLUORESCENCE MINUS ONE CONTROLS

Fluorescence minus one (FMO) controls are used to control nonspecific staining during a multicolor assay. After compensation has been set, a tube containing all of the fluorochrome-labeled antibodies, except one, is run. If the compensation has been properly set, any positive fluorescence in the parameter corresponding to the missing fluorochrome-labeled antibody is caused by nonspecific staining and can be an indication of antibody excess or degradation of related tandem dyes. Although FMO controls are very useful for estimating the sensitivity of a particular detector in the context of other reagents, the controls do not take into account nonspecific binding that can occur with the addition of the test antibody. FMO control tubes are most appropriately used for troubleshooting or when establishing a new multicolor reagent cocktail.

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PROCESS CONTROLS

Process controls, also known as system suitability standards, account for sample preparation and data acquisition. They can include commercially available preserved control cells, cell lines, or primary cells such as normal peripheral blood. Process controls can also be used to test new lots of antibody reagent against old lots.

BIOLOGICAL CONTROLS

When treated or stimulated cells are compared to untreated or unstimulated cells, the untreated or unstimulated cells may in some cases be the most useful control for setting a positive/negative boundary. However, use of isotype controls may also apply to these situations, because stimulation may lead to Fc receptor upregulation, leading in turn to increased background staining, the presence of which can be elucidated by an isotype control.

FLOW CYTOMETRY APPLICATIONS FOR CELLULAR SAMPLES AND CELL THERAPY PRODUCTS

A wide variety of flow cytometry applications have been developed for research, clinical diagnosis and monitoring, drug development, and cellular product characterization and quality assessment (i.e., control to allow batch release). Traditional clinical applications include monitoring HIV disease and diagnosis and monitoring leukemia and lymphoma. Both pharmaceutical and academic research laboratories have increasingly broadened the application of flow cytometry from immunophenotyping to functional cellular assays, as well as microsphere-based multiplex assays capable of measuring multiple functional parameters on individual cells. Current functional assays include those that allow direct study of cellular activation status by measuring intracellular cytokine production or secretion of chemokines or cytokines, using a ligand-binding sandwich assay on microspheres.

Immunophenotyping

Flow cytometry allows the characterization of leukocyte subtypes by labeling cells with fluorochrome-conjugated monoclonal antibodies. The CD system defines monoclonal antibodies that recognize unique cell-surface antigens. Many clinical applications take advantage of flow cytometry's unique capabilities to measure multiple CD antigens on thousands of individual cells.

CD4 ENUMERATION

In the early 1980s, investigators discovered that HIV infects CD4 T cells and that a patient's peripheral blood CD4 T cell count is a useful indicator of immune status. CD4 enumeration has become the most commonly used diagnostic test in HIV-infected patients to determine the need for anti-retroviral (ARV) therapy and for monitoring the effectiveness of ARV drugs. T cell subset counts are typically expressed in terms of cells per microliter and as a percentage of lymphocytes, using a standardized reagent, software, and instrument system.

LEUKEMIA AND LYMPHOMA

Multidimensional flow cytometric analysis enables identification of aberrant cell populations in bone marrow, lymphatic tissue, and peripheral blood of patients with leukemia or lymphoma. This is accomplished with oncology-relevant and lineage-specific cocktails of monoclonal antibodies. With optimal fluorophores and improved optical/electronic configurations in flow cytometry instrumentation, additional cell markers can be detected to more precisely identify leukemia or lymphoma cell phenotypes and to improve the physician's assessment of patient status. Rare-event detection methods have improved the ability to detect minimal residual disease.

DENDRITIC CELLS

Dendritic cells (DCs) act as antigen-presenting cells that can influence the nature and strength of the immune response to specific antigens. This finding has led to the development of DCs as cell-based therapies for cancer, infectious disease, and autoimmune disease. DCs are morphologically and phenotypically diverse and can be derived from several cell types. Two major DC lineages, known as myeloid and plasmacytoid DCs, can be segregated on the basis of their expression of CD11c and CD123, respectively. Additionally, the expression of the costimulatory molecules CD80 and CD86 can be monitored to determine DC maturation state.

STEM AND PROGENITOR CELLS

CD34 expression is commonly used to characterize hematopoietic stem cells (HSCs) in peripheral blood, cord blood, bone marrow, and purified HSC preparations from these sources. Flow cytometric identification and enumeration of HSCs is possible by using monoclonal antibodies specific to the CD34 class III epitope, along with other well-characterized reagents, analysis software, and protocols. The reagent combination of anti-CD45, anti-CD34, and a viability dye such as 7-AAD is widely used for clinical applications. Increasing interest in developing cell-based therapies from embryonic, fetal, and adult tissue sources has led to the use of a wide variety of conventional and novel phenotypic markers for characterization of source cells and their more differentiated progeny. Flow cytometric assays are being developed as part of assay batteries to assess differentiated cellular products derived from pluripotent stem cell sources. These assays will help define appropriate numbers and types of desired cell populations, as well as help detect undesired cells such as residual pluripotent cells that could prove tumorigenic in the recipient.

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LEUKOCYTES

Leukoreduction of blood products is a process used to produce blood products with a residual leukocyte content of less than 5×10^6 per unit. Clinical data suggest that nonhemolytic febrile transfusion reactions can be prevented by leukodepletion. Leukodepletion also prevents alloimmunization to HLA antigens in patients who will repeatedly require transfusion of blood products. Flow cytometry is routinely used to quantitate leukocyte contamination in leukocyte-depleted blood products.

PLATELETS

Flow cytometry is a rapid and useful method for diagnosing many primary thrombocytopathies related to defects in structural or functional glycoproteins (e.g., abnormal expression of gpIIb/IIIa in Glanzmann thrombasthenia or gpIb in Bernard-Soulier disease). The use of thiazole orange, a fluorescent dye that binds RNA, allows immature platelets (reticulated platelets) to be quantified. The reticulated platelet count can be used to determine the rate of thrombopoiesis. This measurement can separate unexplained thrombocytopenias into those with increased destruction and those with defects in platelet production.

ERYTHROCYTES

Rhesus D-negative women receive prophylactic Rh-immunoglobulin to prevent alloimmunization from $Rh(D)^+$ erythrocytes (RBCs). If fetomaternal hemorrhage is suspected, the mother's blood is tested for the presence and quantity of fetal RBCs, using fluorescently labeled antibodies to the Rh(D) antigen or to hemoglobin F.

The reticulocyte count is used to help determine whether the bone marrow is responding adequately to the body's need for RBCs and to help classify different types of anemia. Reticulocyte counts are based on the identification of residual ribosomes and RNA in immature nonnucleated RBCs. Flow cytometric enumeration of reticulocytes and their discrimination from mature RBCs uses fluorescent dyes that bind the residual RNA (e.g., thiazole orange).

Bead-Based Immunoassays

Multiplex microsphere-based flow cytometric assays combine a series of particles of discrete size and/or fluorescence intensity with matched antibody pairs to allow simultaneous detection of multiple soluble analytes on a flow cytometer. The flow cytometer's capacity to discriminate particles on the basis of size and color enables determination of multiple results from a single tube or well. Many investigators use such assays to measure secreted chemokines or cytokines, kinases, and anti-HLA antibodies.

Proliferation Assays

DYE INCORPORATION INTO DNA

Bromodeoxyuridine (BrdU) is a thymidine analog that can be incorporated into the DNA of cells during S phase, and then can be detected using specific, labeled monoclonal antibodies. By pulsing a stimulated cell culture with BrdU, cells can be identified that have proliferated (passed through S phase) during the time of the pulse. This assay has become a useful alternative to ^3H-thymidine incorporation as a measure of proliferation because it is nonradioactive and can identify phenotypes of proliferating cells by the use of multiple markers and flow cytometry.

DYE INCORPORATION INTO CELLULAR PROTEINS OR CELL MEMBRANE

Cell-tracking dyes such as carboxyfluorescein succinimidyl ester (CFSE) and PKH26 have proven useful in the assessment of cell proliferation. CFSE binds covalently to cytosol and membrane proteins, and PKH26 binds non-covalently to cell membranes. When cells divide, CFSE/PKH26 labeling is partitioned equally between the daughter cells, which are therefore half as fluorescent as the parents. The fluorescence of each cell is further halved with each succeeding generation. This property makes CFSE/PKH26 assays useful not only for determining the fraction of cells that have proliferated in a stimulated culture but also, under ideal conditions, the number of generations that have elapsed. In this manner, the precursor frequencies of small populations that have proliferated over several days in culture can be calculated.

Functional Assays

INTRACELLULAR CYTOKINE EXPRESSION

Cell surface and intracellular labeling techniques have been applied to the identification of cell subsets with specific functional characteristics. For example, brief stimulation of cells such as PBMC with protein or peptide antigens can result in the expression of activation markers and cytokines that can then be measured along with other phenotypic markers on the surface of the responding cells. The use of a secretion inhibitor such as brefeldin A or monensin allows the intracellular accumulation of cytokines. The cells are then fixed, permeabilized, and detected by a flow cytometric method. Such assays are useful for monitoring T cell subpopulations that respond to vaccines, infectious disease agents, or cancer. Functional properties of other cell types, including monocytes, DCs, and NK cells, can also be monitored using functional assays with appropriate stimuli.

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KINASES

Phosphorylation-specific cell activation intermediates can be identified using phospho-specific antibodies and flow cytometry. These reagents are useful for mapping intracellular signaling mechanisms, often in the context of other cell-surface phenotypic markers. Thus, multicolor flow cytometry can provide single-cell assessment of intracellular activation states in complex cell populations. These assays may have utility in detecting altered signaling states in cancer cells or in directing appropriate therapies based on the signaling properties of a patient's tumor cells.

APOPTOSIS

Apoptosis, commonly described as programmed cell death, is the process of cell death caused by regulated, physiologic processes. The apoptotic process manifests itself as a series of morphological, biochemical, and molecular changes to the cell and can be initiated by external or internal stimuli. A central event during apoptosis is the activation of caspases, a family of proteolytic enzymes. Caspases are synthesized as inactive proenzymes and are activated by other caspases or by similar molecules. They form a cascade that can lead to the cleavage of various cytoplasmic or nuclear proteins. One of the caspases that is reported to be crucial for the apoptotic process is caspase-3, which is activated during the early stages of apoptosis.

Flow cytometric methods for detecting apoptotic cells include measuring morphology, changes in membrane structure, DNA cleavage by endonucleases, and mitochondrial membrane potential. Natural or artificial caspase substrates or antibodies against the activated form of the enzyme have also been used for this purpose.

CELL VIABILITY

Flow cytometry is often used to discriminate live cells from dead cells. The principle of nucleic acid dye exclusion is the basis of this application. A nucleic acid dye such as PI or 7-AAD is added to cells in suspension. During flow cytometric analysis, cells that fluoresce above background are considered nonviable because they cannot exclude the dye, which fluoresces when it binds to cellular DNA.

Flow Cytometry Immunoglobulin Assays

FLOW CYTOMETRY CROSS-MATCHING

Before organ transplantation, flow cytometry cross-matching (FCXM) is performed on recipients to screen for anti-HLA antibodies that can cause rejection. Anti-HLA antibodies are detected by incubating HLA-defined leukocytes, B-cell lines, or HLA antigen-coated beads with the serum sample, followed by anti-human immunoglobulin fluorescently labeled antibodies. Leukocytes are immunostained to identify T and B cells in order to distinguish between anti-HLA class I and II activity, respectively. In addition, screening of blood donations for anti-HLA antibodies is also increasingly employed to identify donors whose blood products may have increased risk of causing transfusion-related acute lung injury (TRALI) in recipients.

ANTI-HUMAN NEUTROPHIL ANTIBODIES

Anti-human neutrophil antibodies (HNA) can cause neutropenia and have been implicated in TRALI. Autoimmune neutropenias may develop in patients who have autoimmune disorders such as Felty syndrome, systemic lupus erythematosus, and Hashimoto thyroiditis. The absence of anti-HNA antibodies narrows the differential diagnosis to nonimmune causes such as bone marrow failure, myelodysplasia, or marrow-infiltrative processes. Flow cytometry can detect anti-neutrophil antibodies and can confirm the origin of neutropenia or TRALI.

ANTI-HUMAN PLATELET ANTIBODIES

Anti-human platelet antibodies (HPA) are detected by both indirect and direct flow cytometry-based platelet-associated immunoglobulin assays. In autoimmune thrombocytopenic purpura, free serum antibodies are not found as frequently as are platelet-bound antibodies. In cases of alloantibody formation, serum antibodies may be detected without evidence of platelet-associated antibodies.

FLOW CYTOMETRY ASSAY TROUBLESHOOTING

When developing a flow cytometry method, first determine the ultimate purpose of the assay. For assays intended for research, the cell samples, reagents, and protocols may be difficult to standardize. Assays intended for patient diagnosis or to qualify a cellular product for release before administration to a patient demand more stringent assay and sample standardization. Regulatory guidelines, the type and stage of clinical investigation, and the ultimate purpose of the assay determine the level of assay rigor required.

Flow cytometry assay development should include the establishment and qualification of staining, handling, instrument, and analysis parameters and limits. Assuming that the method has been well developed, the operators are properly trained, the instrument has been properly set up, appropriate assay and instrument controls have been applied, and, if necessary, the instrument and method validations have been performed, operators may encounter and address instances when troubleshooting is necessary.

The most common flow cytometry challenges are high fluorescence or side scatter background, abnormal event rates, high fluorescence intensity, and low fluorescence signal. Approaches to alleviating these issues are described below.

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High Particulate Background

Excessive cell handling (e.g., vortexing), improper fixation, and bacterial contamination of the cells can all increase the particulate background. In addition, if the instrument's forward threshold is set too low, cell debris will be detected as events. Gentle cell handling, fresh reagents, and appropriate instrument settings help ensure consistent side scatter profiles.

High Fluorescence Background

High fluorescence intensity can be attributed to excessive antibody concentration, inadequate cell washing, or inadequate Fc receptor blocking. In addition, improperly high instrument PMT gain can also result in a high background. Consistent antibody concentration and cell density, adequate washing and blocking, and appropriate instrument settings will help avoid abnormally high fluorescence background.

High Event Rate

Abnormally high event rates are often attributed to high cell densities during antibody staining or in the final cell sample. Inadequate mixing and settling of the cell sample can result in high cell event rates, as can improper or inconsistent gating.

Low Event Rate

Cell clumping, low final sample cell densities, blockages in the instrument fluidics, or improper gating can often result in abnormally low event detection. Proper cleaning, maintenance, and setup of the instrument, as well as consistent staining protocols, can help achieve consistent results with sufficient sensitivity.

High Fluorescence Intensity

As in the case for high fluorescence background, high mean cell fluorescence can result from too much labeled antibody, inadequate or inconsistent cell washing, or inadequate blocking. Including detergent in the wash buffer, especially during intracellular staining, can help prevent nonspecific antibody binding.

Weak Fluorescence Intensity

Many factors can result in weak fluorescence intensity. Instrument parameters such as poor laser alignment, improper compensation, improper setup, inconsistent gain settings, and weak laser output can all negatively affect fluorescence intensity. In addition, cell physiology or reagent preparation issues, such as insufficient antibody concentration, labile or secreted target antigen, poor-quality or improperly stored reagents (resulting in fluorochrome fading), or inaccessible target antigen, can all result in a weak signal. Adequate assay development, proper instrument maintenance, and adherence to qualified protocols can all improve the fluorescence signal intensity.

Flow cytometry enables investigators to analyze cells for many different applications. Types of immunophenotypic and functional assays are increasing in number and in scope. The presence of proteins and cellular processes and detection of rare or abnormal cell populations can be studied. The reader is referred to the technical literature for application and method details.

REFERENCES

Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. *Current Protocols in Immunology*. Fourth ed. Hoboken, NJ: John Wiley and Sons; 1994.

Shapiro HM. *Practical Flow Cytometry*. Fourth ed. Hoboken, NJ; John Wiley and Sons; 2003.

⟨1029⟩ GOOD DOCUMENTATION GUIDELINES

INTRODUCTION

Purpose

Documentation can be viewed as the foundation of all quality systems because clear, complete, accurate records are essential to all operations and procedures. This general chapter provides guidelines on good documentation practices for the Good Manufacturing Practice (GMP)-regulated industries, to be used in the production and control of pharmaceutical products, active pharmaceutical ingredients (APIs), excipients, dietary supplements, food ingredients, and medical devices. This chapter describes the underlying principles of proper documentation for GMP operations to assist the user while working with GMP activities. These guidelines should be helpful for building the basic foundation of a quality system that will ensure proper documentation as well as record integrity and control.

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Scope

This chapter covers different levels and types of documentation, including paper and electronic records that consist of raw data, reports, protocols, and procedures related to manufacturing controls and analytical data. The chapter also includes recommendations on information that should be recorded for various types of GMP documents. Electronic systems should be developed to meet guidelines described in this chapter.

This chapter does not provide information about all applicable current legal requirements, nor does it affect any applicable current requirements under GMP regulations.

PRINCIPLES OF GOOD DOCUMENTATION

All steps related to the manufacturing, testing, packing, or holding of pharmaceutical products, APIs, excipients, dietary supplements, food ingredients, and medical devices should be documented.

Good documentation principles for manual or electronic records include the following, as applicable:

- Records should be clear, concise, accurate, and legible.
- Data entries should be recorded promptly when actions are performed.
- Backdating and postdating are not allowed.
- All corrections to the original entries should be initialed and dated (or captured within an electronic audit trail), with an explanation included in cases where the reason for the change is not obvious.
- Data entries should be traceable to the person who made the entry.
- Uncommon abbreviations and acronyms should be defined.
- Controls should be in place to protect the integrity of the records.
- In the event that ink may have faded over time (e.g., thermal paper), a copy can be used with verification of its accuracy; the copy should be initialed and dated.
- Notebooks, data sheets, and worksheets should be traceable.
- An adequate documentation system is needed to ensure data integrity and availability of current and archived records.
- Records should be retained per regulatory requirements and be readable during the retention period.
- All pages should be paginated. Attachments (supporting documents) should be paginated with a reference to the parent document.

DATA COLLECTION AND RECORDING

Formats for data collection and recording include, but are not limited to, the following:

- Paper forms, data sheets, and worksheets
- Notebooks and logbooks
- Instrument printouts
- Electronic data obtained with a system such as an electronic data system, laboratory information management system (LIMS), or electronic laboratory notebook (ELN)

All data should be permanently recorded directly and legibly when the activity is performed. If it is paper record, then it should be recorded in indelible ink. All data entries should be traceable to who made the entry and when. Additionally, electronic records must meet the requirements of the *Code of Federal Regulations Title 21* (21 CFR), Part 11.

Any change to an entry should be made in a way that does not obscure the original entry, with an explanation in cases where the reason for the change is not obvious. Changes should be traceable to who made the change and when the change was made. For clarity, predefined correction codes may be used, for example, WD = wrong date.

Notebook pages and worksheets should be used consecutively, and information should be recorded chronologically. GMP records such as batch records, test methods, and specifications should be given unique identifiers and use version control for the documents.

All data entry fields should be completed. A single line and/or "N/A" should be drawn through portion(s) of a page that are not used. If the record is in an electronic system and the system provides traceability of who filled each field and when, the field can be left blank.

Decimals less than one should be preceded by a zero. Rounding rules and guidelines on significant figures are described in *General Notices, 7.20 Rounding Rules*.

All dates should be expressed in a format that clearly indicates the day, month, and year.

All GMP records for data collection should undergo appropriate review and signature by a second person to confirm the accuracy, compliance, and completeness of the entries. Additional signatures may be required on the basis of local Standard Operating Procedures (SOPs) for different levels of review (e.g., performed by, verified by, checked by, reviewed by, approved by) as accountability steps.

An official record of signature and initials should exist for each employee or can be contained within the document. Controls should be in place for assigning signature approval requirements and delegation of signature authority, when needed.

In the event that verified copies of raw data have been prepared, the verified copy may be substituted for the original source as raw data.

All multiple-page data sheets or instrument printouts in paper form should be signed/initialed on the first or last page with a note indicating the total number of pages. The first page and all subsequent pages should be uniquely identified to the activity being performed, such as the notebook reference, study number, or worksheet reference.

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DIFFERENT TYPES OF GMP DOCUMENTS

The following or similar documents should include the following information, but are not limited to these items.

Laboratory Records

Laboratory records should be organized to ensure that the records are concise, clear, legible, and accurate, and detail the following:

- Description of materials, such as reagents. This information typically includes the material name, manufacturer and lot number, titer or concentration, expiration date, grade (if known), and a reference to the lab notebook if prepared in the lab
- Identification of equipment used. This information typically includes equipment name, unique control number, and calibration expiration date, as applicable
- Procedures used
- Measurements
- Formulae and calculations
- Results and conclusions

Equipment-Related Documentation

All equipment used in manufacturing, testing, packing, or holding of a raw material, component, API, finished product, or other similar item should be maintained and qualified for its intended use. The documentation related to equipment includes:

- Policies and procedures for operation and maintenance
- Equipment use
- Maintenance records
- Calibration or qualification records
- Instrument labeling

Deviations and Investigations

All aberrations, anomalies, and exceptions related to manufacturing, testing, packing, or holding of a raw material, component, API, finished product, or other similar item should be documented. Once documented, the deviation should be evaluated and investigated, as appropriate. Procedures should be in place for documenting, evaluating, and investigating such events. Documentation of the investigation should include the following:

- Description of the event
- Root-cause investigation
- Evaluation of data trend
- Responsibilities of people involved in the investigation or deviations
- Impact assessment
- Corrective Action and Preventive Action (CAPA) with timelines
- Review and approval

Batch Records

A Master Batch Record (MBR) is created as a template for the manufacture of a specific product. An Executed Batch Record, based on the MBR, is used to document the steps and materials involved in the production of a specific batch of a raw material, component, API, finished product, or other similar item. Typically, the following sections are included in a Batch Record, and should be approved by an appropriate representative from the manufacturing site or packaging site:

- Header information (e.g., product name, batch number, manufacturing site)
- Unit of operation (e.g., blending, coating, filling)
- Manufacturing process
  - Target weights (raw materials)
  - Conditions (time, temperature)
  - Deviations and investigations
- In-process sampling or testing
- Other critical information, as applicable
- Sampling plan for release, stability, and retention
- Review and approval

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Certificate of Analysis

The purpose of the Certificate of Analysis ("C of A" or CoA) is to report analytical results for a specific batch of a raw material, component, API, finished product, or other similar item. Typically, the following sections are included on a C of A and should be approved by an appropriate representative from the testing site:

- Vendor, supplier, or manufacturer information (as applicable)
- Product information (name and strength)
- Results for the specific batch, with name of test, acceptance criteria, and result for each test
- Conformance statement or equivalent
- Reference to procedure and specification document
- Reference of data source
- Approval and date
- Expiration date or retest information

Standard Operating Procedures

The purpose of an SOP is to provide directions to trained personnel regarding a given set of activities. SOPs should be clear and concise. The following sections are typically included in an SOP:

- Purpose and scope
- Instructions and procedure
- Responsibilities and roles
- Materials or equipment, as appropriate
- Definitions or references, as needed
- Review and approval
- Revision history

Protocols and Reports

Many tasks and activities are executed on the basis of a predefined, preapproved protocol. The results of these activities are then documented in a final report with conclusions. Examples of such activities are as follows:

- Equipment qualification
- Analytical method validation or verification
- Manufacturing process validation
- Analytical method or manufacturing technology transfer
- Cleaning validation
- Stability study or testing
- Comparability study

Both the protocol and the report should typically include the following sections:

- Purpose
- Plan or instructions
- Predetermined acceptance criteria
- Deviations or investigations, or a reference to (for report only)
- Assessment or evaluation (for report only)
- Data reference (for report only)
- Review and approval
- Revision history

Analytical Procedures

Analytical procedures provide direction to an operator on how to perform a given analytical test. The following sections will typically be included in the analytical procedure:

- Purpose
  - Test information
  - Product information
- Safety information, if applicable
- Materials and equipment
- Procedure, as applicable
  - System suitability
  - Preparation of solutions and reagents
  - Preparation of standards and samples
  - Instrument parameters

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- Calculations and reporting
- Review and approval with approval dates
- Revision history

Training Documentation

Personnel should be trained to perform their assigned tasks. The training should be documented, and the training records should be retained and kept readily accessible. In general, training documentation should include:

- Training description including name of training, version, and mode (self-training or instructor led)
- Completion date
- Information on the trainer, as applicable

Retention of Documents

An adequate policy for record retention and archiving should be established for the above records. The required length of time depends on the regulatory requirements or company procedures; however, it should be at least 1 year after the batch expiration date.

⟨1030⟩ BIOLOGICAL ASSAY CHAPTERS—OVERVIEW AND GLOSSARY

USP–NF contains four general chapters regarding the development, validation, and analysis of bioassays (biological assays): *Design and Analysis of Biological Assays* ⟨111⟩, *Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩. This proposed new chapter, *Biological Assay Chapters—Overview and Glossary* ⟨1030⟩, provides an overview and some material common to chapters ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩, including a glossary of bioassay-related terms.

The suite of *USP* bioassay chapters focuses on relative potency assays. These assays recognize the inherent variability in biological test systems (whether animals or cells) that may be seen from laboratory to laboratory and from day to day. That inherent variability compromises the reliability of an absolute measure of potency. In relative potency assays, the biological activity of a Test material is compared to the activity of a Standard in an assay system wherein the use of a Standard reduces the influence of the inherent variability of the system on the estimation of relative potency. Relative potency assays also provide focus on important variability in response because of differences between the Test and Standard materials (if such a difference exists). The Test is expected to behave as a dilution or concentration of the Standard and should exhibit the property of *similarity*. Although they are intended for relative potency bioassays, the principles and practices developed in these chapters may have wider application—for example, to immunoassays and receptor-ligand–binding assays used to determine relative potency.

Chapter ⟨1032⟩ provides information for scientists developing a new biological assay. As seen in *Table 1*, the chapter covers a range of activities across the life cycle of the assay, with emphasis on development leading to validation, including the choice of test system and design considerations (e.g., plate layout). It also addresses data analysis strategies that should be considered during development (before validation) but that are not routinely addressed later. Among these strategies are the choice of weighting scheme, data transformation, if any, and choice of statistical model. Statistical details in support of these sections of chapter ⟨1032⟩ are found in chapter ⟨1034⟩.

Table 1. Primary Sections of *Design and Development of Biological Assays* ⟨1032⟩

Section	Section Title
1	Introduction
1.1	Purpose and Scope
1.2	Audience
2	Bioassay Fitness for Use
2.1	Process Development
2.2	Process Characterization
2.3	Product Release
2.4	Process Intermediates
2.5	Stability
2.6	Qualification of Reagents
2.7	Product Integrity
3	Bioassay Fundamentals
3.1	In Vivo Bioassays
3.2	Ex Vivo Bioassays

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Table 1. Primary Sections of *Design and Development of Biological Assays* ⟨1032⟩ *(continued)*

Section	Section Title
3.3	In Vitro (Cell-Based) Bioassays
3.4	Standard
4	Statistical Aspects of Bioassay Fundamentals
4.1	Data
4.2	Assumptions
4.3	Variance Heterogeneity, Weighting, and Transformation
4.4	Normality
4.5	Linearity of Concentration–Response Data
4.6	Common Bioassay Models
4.7	Suitability Testing
4.8	Outliers
4.9	Fixed and Random Effects in Models of Bioassay Response
5	Stages in the Bioassay Development Process
5.1	Design: Assay Layout, Blocking, and Randomization
5.2	Development
5.3	Data Analysis during Assay Development
5.4	Bioassay Validation
5.5	Bioassay Maintenance

Chapter ⟨1034⟩ provides information about the data analyses appropriate for common relative potency bioassays, including parallel-line, slope-ratio, parallel-curve, and quantal models. The chapter also includes analyses supporting system and sample suitability assessment and methods for combining results from independent assays (see *Table 2*). This chapter is the most statistically advanced of the three chapters but is designed to be suitable for both biologists and statisticians. The conceptual material requires only a minimal statistical background. Methods sections require a statistical background at the level of *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and familiarity with linear regression.

Table 2. Primary Sections of *Analysis of Biological Assays* ⟨1034⟩

Section	Section Title
1	Introduction
2	Overview of Analysis of Bioassay Data
3	Analysis Models
3.1	Quantitative and Qualitative Assay Responses
3.2	Overview of Models for Quantitative Responses
3.3	Parallel-Line Models for Quantitative Responses
3.4	Nonlinear Models for Quantitative Responses
3.5	Slope–Ratio Concentration–Response Models
3.6	Dichotomous (Quantal) Assays
4	Confidence Intervals
4.1	Combining Results from Multiple Assays
4.2	Combining Independent Assays (Sample-Based Confidence Interval Methods)
4.3	Model-Based Methods
5	Additional Sources of Information

Chapter ⟨1033⟩ is intended to follow chapters ⟨1032⟩ (assay development) and ⟨1034⟩ (development of data analysis plans). That is, chapter ⟨1033⟩ assumes a fully developed bioassay (including a data analysis plan and at least tentative values for system and sample suitability criteria and the bioassay format) and provides guidance about the validation of that assay. The chapter addresses the validation characteristics relevant to relative potency bioassays and provides more detail regarding the statistical methods used in validation than does *Validation of Compendial Procedures* ⟨1225⟩. Principles and practices developed in chapter

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⟨1033⟩, although they are intended for relative potency assays, may have wider application. The chapter emphasizes validation approaches that provide flexibility in adopting new bioassay methods, new biological drug products, or both (see *Table 3*).

Table 3. Primary Sections of *Biological Assay Validation* ⟨1033⟩

Section	Section Title
1	Introduction
2	Fundamentals of Bioassay Validation
2.1	Bioassay Validation Protocol
2.2	Documentation of Bioassay Validation Results
2.3	Bioassay Validation Design
2.4	Validation Strategies for Bioassay Performance Characteristics
2.5	Validation Target Acceptance Criteria
2.6	Assay Maintenance
2.7	Statistical Considerations
3	A Bioassay Validation Example
3.1	Intermediate Precision
3.2	Relative Accuracy
3.3	Range
3.4	Use of Validation Results for Bioassay Characterization
3.5	Confirmation of Intermediate Precision and Revalidation
4	Additional Sources of Information
Appendix	Measures of Location and Spread for Lognormally Distributed Variables

GLOSSARY

This glossary pertains to biological assays and provides a compendial perspective that is consistent across *USP–NF*'s suite of bioassay chapters, is complementary to previous authoritative usage, and provides a useful focus on the bioassay context. In many cases the terms cited here have common, though undocumented, usages or are defined in *Validation of Compendial Procedures* ⟨1225⟩ and in the International Conference on Harmonization (ICH) Guideline Q2(R1), *Validation of Analytical Procedures: Text and Methodology*.[1] (Chapter ⟨1225⟩ and ICH Q2(R1) agree on definitions.) The *Glossary* is intended to be consistent with these precedent usages, and notes are provided when a difference arises because of the bioassay context. Definitions from ⟨1225⟩ and ICH Q2(R1) are identified as, for example, " ⟨1225⟩" if taken without modification, or "adapted from ⟨1225⟩" if taken with minor modification for application to bioassay. Most definitions are accompanied by notes that elaborate on the bioassay context.

The terms are organized alphabetically within five topic sections:

I. General terms related to bioassays
II. Terms related to performing a bioassay
III. Terms related to precision and accuracy
IV. Terms related to validation
V. Terms related to statistical design and analysis

Table 4 shows each term and the *Glossary* section in which it can be found.

Table 4. Terms Listed in the *Glossary*

Term	Section	Term	Section
Accuracy	III	Mixed-effects model	V
Analysis of variance (ANOVA)	V	Modeling, statistical	V
Analytical procedure	I	Nested	V
Assay	I	Out of specification	II
Assay data set	I	Parallelism	V
Bioassay	I	Partially crossed	V

[1] Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. Accessed 29 March 2012.

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Table 4. Terms Listed in the *Glossary* *(continued)*

Term	Section	Term	Section
Biological assay	I	Point estimate	V
Blocking	V	Potency	I
Complete block design	V	Precision	III
Confidence interval	V	Pseudoreplication	V
Crossed	V	*P* value	V
Design of experiments (DOE)	V	Quantitation limit	IV
Dilutional linearity	IV	Random effect	V
Direct bioassays	I	Random error	III
Equivalence test	V	Random factor	V
Errors, types of	III	Randomization	V
Expected mean square	V	Range	IV
Experimental design	V	Relative bias	III
Experimental unit	V	Relative potency	I
Factor	V	Repeatability	III
Factorial design	V	Replication	V
Fixed effect	V	Reportable value	I
Fixed factor	V	Reproducibility	III
Format variability	III	Robustness	IV
Format, bioassay	II	Run	I
Fractional factorial design	V	Sample suitability	II
Full factorial design	V	Significance probability	V
General linear model	V	Similar preparations	I
Geometric coefficient of variation	III	Similarity (algebraic)	I
Geometric standard deviation	III	Specificity	III
Incomplete block design	V	Standard error of estimate	V
Independence	V	Statistical process control (SPC)	V
Indirect bioassays	I	System suitability	II
Interaction	V	Systematic error	III
Intermediate precision	III	True replicates	V
Level	V	Truncation bias	III
Linearity, dilutional	IV	Type I error	V
Lognormal distribution	V	Type II error	V
Lower limit of quantitation	IV	Validation, assay	IV
Mean square	V	Variance component analysis	V

I. General Terms Related to Bioassays

ANALYTICAL PROCEDURE [ADAPTED FROM Q2(R1)]

Detailed description of the steps necessary to perform the analysis.

[NOTE—1. The procedure may include but is not limited to the sample preparation, the Reference Standard, and the reagents; use of equipment; generation of the standard curve; use of the formulae for the calculation; etc. 2. An FDA Guidance[2] provides a list of information that typically should be included in the description of an analytical procedure.]

[2] Analytical Procedures and Methods Validation for Drugs and Biologics, Guidance for Industry. 2015. http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm386366.pdf. Accessed 20 April 2016.

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ASSAY

Analytical procedure to determine the quantity of one or more components or the presence or absence of one or more components.

[NOTE—1. *Assay* often is used as a verb synonymous with *test* or *evaluate*, as in "I will assay the material for impurities." In this glossary, *assay* is a noun and is synonymous with *analytical procedure* (q.v.). 2. The phrase *to run the assay* means to perform the analytical procedure as specified. 3. In common practice, *assay* and *run* (q.v.) often are used interchangeably. In this glossary, they are different. Also see *bioassay* and *bioassay data set*.]

ASSAY DATA SET

The set of data used to determine a single potency or relative potency for all samples included in the bioassay.

[NOTE—1. The definition of an assay data set can be subject to interpretation as necessarily a minimal set. It may be possible to determine a potency or relative potency from a set of data but not do this well. It is not the intent of this definition to mean that an assay data set is the minimal set of data that can be used to determine a relative potency. In practice, an assay data set should include, at least, sufficient data to assess similarity (q.v.). It also may include sufficient data to assess other assumptions. 2. It is also not an implication of this definition that assay data sets used together in determining a reportable value (q.v.) are necessarily independent from one another, although it may be desirable that they be so. When a run (q.v.) consists of multiple assay data sets, independence of assay sets within the run must be evaluated.]

BIOASSAY, BIOLOGICAL ASSAY (these terms are interchangeable)

Analysis (as of a drug) to quantify the biological activity or activities of one or more components by determining its capacity for producing an expected biological activity on a culture of living cells (in vitro) or on test organisms (in vivo), expressed in terms of units.

[NOTE—1. The components of a bioassay include the analytical procedure, the statistical design for collecting data, and the method of statistical analysis that eventually yields the estimated potency or relative potency. 2. Bioassays can be either direct or indirect.

Direct bioassays—Bioassays that measure the concentration of a substance that is required in order to elicit a specific response. For example, the potency of digitalis can be directly estimated from the concentration required to stop a cat's heart. In a direct assay, the response must be distinct and unambiguous. The substance must be administered in such a manner that the exact amount (threshold concentration) needed to elicit a response can be readily measured and recorded.

Indirect bioassays—Bioassays that compare the magnitude of responses for nominally equal concentrations of reference and test preparations rather than test and reference concentrations that are required to achieve a specified response. Most biological assays in *USP–NF* are indirect assays that are based on either quantitative or quantal (yes/no) responses.]

POTENCY [21 CFR 600.3(S)]

The specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.

[NOTE—1. A wholly impotent sample has no capacity to produce the expected specific response, as a potent sample would. Equipotent samples produce equal responses at equal dosages. Potency typically is measured relative to a Reference Standard or preparation that has been assigned a single unique value (e.g., 100.0) for the assay; see *relative potency*. At times, additional qualifiers are used to indicate the physical standard employed (e.g., "international units"). 2. Some biological products have multiple uses and multiple assays. For such products there may be different reference lots that do not have consistently ordered responses across a collection of different relevant assays. 3. [21 CFR 610.10] Tests for potency shall consist of either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in 21 CFR 600.3(s).]

RELATIVE POTENCY

A measure obtained from the comparison of a Test to a Standard on the basis of capacity to produce the expected potency.

[NOTE—1. A frequently invoked perspective is that relative potency is the degree to which the Test preparation is diluted or concentrated relative to the Standard. 2. Relative potency is unitless and is given definition, for any test material, solely in relation to the reference material and the assay.]

REPORTABLE VALUE

The value that will be compared to an acceptance criterion.

[NOTE—1. The acceptance criterion for comparison may be in the *USP* monograph, or it may be set by the company, e.g., for product release. 2. The term *reportable value* is inextricably linked to the "intended use" of an analytical procedure. Assays are performed on samples in order to yield results that can be used to evaluate some parameter. Assays may have different summary values or formats for different purposes (e.g., lot release vs. calibration of a new reference standard). The reportable value may be different even if the mechanics of the test itself are identical. Validation is required in order to support the properties of each choice of reportable value. In practice there may be one physical document that is the analytical procedure used for more than one application, but each application must be detailed separately within that document. Alternatively, there may be separate documents for each application. 3. When the inherent variability of a biological response, or that of the log potency, precludes a single assay data set's attaining a value sufficiently accurate and precise to meet a specification, the assay format

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may be changed as necessary. The number of blocks or complete replicates needed depends on the assay's inherent accuracy and precision and on the intended use of the reported value. It is practical to improve the precision of a reported value by reporting the geometric mean potency from multiple assays. The number of assays used is determined by the relationship between the precision required for the intended use and the inherent precision of the assay system.]

RUN

The performance of the analytical procedure that can be expected to have consistent precision and trueness; usually, the assay work that can be accomplished by a single analyst in a set time with a given unique set of assay factors (e.g., standard preparations).

[NOTE—1. There is no necessary relationship of run to assay data set (q.v.). The term *run* is laboratory specific and relates to the laboratory's physical capability and environment for performing the work of an assay. An example of a run is given by one analyst's simultaneous assay of several samples in one day's bench work. During the course of a single run, it may be possible to determine multiple reportable values. Conversely, a single assay data set may include data from multiple runs. 2. From a statistical viewpoint, a run is one realization of the factors associated with intermediate precision (q.v.). Within-run variability is thus repeatability. It is good practice to associate runs with factors that are significant sources of variation in the assay. For example, if cell passage number is an important source of variation in the assay response obtained, then each change in cell passage number initiates a new run. If the variance associated with all factors that could be assigned to runs is negligible, then the influence of runs can be ignored in the analysis, and the analysis can focus on combining independent analysis data sets. 3. When a run contains multiple assays, caution is required regarding the independence of the assay results. Factors that typically are associated with runs and that cause lack of independence include cell preparations, groups of animals, analyst, day, a common preparation of reference material, and analysis with other data from the same run. Even though a strict sense of independence may be violated because some elements are shared among the assay sets within a run, the degree to which independence is compromised may have negligible influence on the reportable values obtained and should be verified and monitored.]

SIMILAR PREPARATIONS

The property that the Test and the Standard contain the same effective constituent, or the same effective constituents in fixed proportions, and all other constituents are without effect in some specific assay context.

[NOTE—1. Having similar preparations is often summarized as the property that the Test behaves as a dilution (or concentration) of the Standard. 2. Similar preparations are fundamental to methods for determination of relative potency. Given similar preparations, a relative potency can be calculated, reported, and interpreted. In the absence of similar preparations, a meaningful relative potency cannot be reported or interpreted. 3. The practical consequence of similar preparations is algebraic similarity (q.v.). (Also see *Parallelism, section V.*)]

SIMILARITY (ALGEBRAIC)

The Test and Standard concentration-response curves are algebraically related in a manner consistent with similar preparations.

[NOTE—1. Examples of similarity are parallelism (q.v.) of concentration–response curves and equality of intercepts in slope ratio models. 2. Failure to statistically demonstrate dissimilarity between a Reference and a Test does not amount to demonstration of similarity. To demonstrate similarity an equivalence approach is appropriate; see ⟨1032⟩ and ⟨1034⟩. 3. Similarity is typically a sample suitability (q.v.) criterion. Note, however, that suitability is a necessary but not sufficient condition for preparations to be similar. In practice, absent knowledge of differences between the Test and Standard materials, demonstration of similarity is accepted as demonstrating similar preparations.]

II. Terms Related to Performing a Bioassay

FORMAT, BIOASSAY

The intra- and inter-run replication strategy for replication of assay data sets that has been determined by variance analysis to support the use of the bioassay.

[NOTE—1. Modifications to bioassay format may occur as new information regarding sources of variability becomes available. Such modifications do not include changes to the dilution scheme of Test samples or Standard, or the replication strategy (part of what is sometimes called bioassay configuration). Assay configuration can include nested dimensions like plate design, multiple plates per day, single plates on multiple days, etc. 2. The geometric mean relative potency determined from the bioassay format is the reportable value, which may be used to assess conformance to specifications or as a component of subsequent analysis (e.g., stability evaluation).]

OUT OF SPECIFICATION (OOS)

The property of a reportable value that falls outside its specification acceptance criterion.

[NOTE—*Out of specification* is not a property of the bioassay but rather a property of Test samples. The term is introduced into ⟨1033⟩ in conjunction with setting validation acceptance criteria which limit the risk of producing out-of-specification test results because of bioassay performance characteristics.]

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SAMPLE SUITABILITY

A sample is suitable (may be used in the estimation of potency) if its response curve satisfies limits on critical properties that are stated in the assay procedure.

[NOTE—Response curve properties are to be taken generally; i.e., includes outliers and variability. The most significant of these properties for bioassays is similarity (q.v.) to the standard response curve. In addition, all assay systems have limits on the range of values they can report. For samples that fail one or more sample suitability criteria in a bioassay, the potency estimate from those samples should not be used as a reportable value or as a contributor to a reportable value. Also see *truncation bias* in this *Glossary* and the sections *Sample Suitability* and *Range* in general chapter ⟨1032⟩.]

SYSTEM SUITABILITY

An assay system is suitable for its intended purpose if it is capable of providing legitimate measurements as defined in the assay protocol.

[NOTE—System suitability may be thought of as an assessment of whether there is any evidence of a problem in the assay system. An example is provided by positive and negative controls, where values outside their normal ranges suggest that the assay system is not working properly.]

III. Terms Related to Precision and Accuracy

ACCURACY ⟨1225⟩

The closeness of test results obtained by the procedure and the true value.

[NOTE—1. ICH and USP give the same definition of accuracy. However, ISO specifically regards accuracy as having two components, bias and precision.[3] That is, to be *accurate* as used by ISO, a measurement must both be on target (have low bias) and be precise. In contrast, ICH Q2(R1) states that accuracy is sometimes termed "trueness" but does not define *trueness*. ISO defines *trueness* as the "closeness of agreement between the average value obtained from a large series of test results and an accepted reference value" and indicates that "trueness is usually expressed in terms of bias." The 2001 FDA Guidance on Bioanalytical Method Validation[4] defines *accuracy* in terms of "closeness of *mean* test results obtained by the method to the true value (concentration) of the analyte" (emphasis added) and thus is consistent with the ICH usage. This glossary adopts the USP/ICH approach. That is, *accuracy* is defined as the agreement between the mean (or expected results) from an assay and the true value, and uses the phrase *accurate and precise* to indicate low bias (accurate) and low variability (precise). 2. Considerable caution is needed when using or reading the term *accuracy*. In addition to the inconsistency between USP/ICH and ISO, common usage is not consistent. 3. For purposes of bioassay validation, the terms *accuracy* and *bias* have been replaced by *relative accuracy* and *relative bias*.]

ERROR, TYPES OF

Two sources of errors that affect the uncertainty of results of a biological assay are systematic error and random error.

A **systematic error** is one that happens with similar magnitude and consistent direction repeatedly. This introduces a bias in the determination. Effective experimental design, including randomization and/or blocking, can reduce systematic error.

A **random error** is one whose magnitude and direction vary without pattern. Random error is an inherent variability or uncertainty of the determination. Conversion of systematic into random error, through experimental design or randomization, increases the robustness of a biological assay and allows a comparatively simple analysis of assay data but may require a larger sample size.

FORMAT VARIABILITY

Predicted variability for a particular bioassay format.

GEOMETRIC COEFFICIENT OF VARIATION

Found as antilog(S)−1, where S is the standard deviation determined in the log scale.

[NOTE—The geometric coefficient of variation is usually reported as a percentage (%GCV). It is important not to confuse the %GCV with the %CV. The %GCV is a measure of spread relevant to data analyzed in the log-transformed [Y = log(X)] scale, and the %CV is a measure relevant to data analyzed in the original (X) scale.]

GEOMETRIC STANDARD DEVIATION (GSD)

The variability of the log-transformed values of a lognormal response expressed as a percentage in the untransformed scale. It is found as antilog(S), where S is the standard deviation determined in the log scale.

[NOTE—For example, if the standard deviation of log potency is S using log base 2, the GSD of potency is $100 * 2^S$.]

[3] ISO. International Standard 5725-1. Accuracy (Trueness and Precision) of Measurement Methods and Results—Part 1: General Principles and Definitions. Geneva, Switzerland; 1994.

[4] FDA. Guidance for Industry. Bioanalytical Method Validation. May 2001. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf. Accessed 7 December 2011.

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INTERMEDIATE PRECISION (ADAPTED FROM ⟨1225⟩)

Within-laboratory precision associated with changes in operating conditions.

[NOTE—1. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay, including different days, different analysts, different equipment, etc. Intermediate precision is thus "intermediate" in scope between the extremes of repeatability (intra-assay) and reproducibility (inter-laboratory). 2. Any statement of intermediate precision should identify the factors that varied. For example, "The intermediate precision associated with changing equipment and operators is...." 3. Investigators can benefit from separately identifying the precision associated with each source (e.g., inter-analyst precision). This may be part of assay development and validation when there is value in identifying the important contributors to intermediate precision. 4. When reporting intermediate precision, particularly for individual sources, care should be taken to distinguish between intermediate precision variance and components of that variance. The intermediate precision *variance* includes repeatability and thus must be at least as large as the repeatability variance. A variance *component*, e.g., associated with analyst, also is a part of the intermediate precision variance for analyst, but it could be negligible and need not be larger in magnitude than the repeatability variance.]

PRECISION (⟨1225⟩)

Measure of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample.

[NOTE—1. Precision may be considered at three levels: repeatability (q.v.), intermediate precision (q.v.), and reproducibility (q.v.). 2. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample, precision can be investigated using spiked samples that mimic a true sample or a sample solution. 3. Precision may be expressed as the variance, standard deviation, coefficient of variation, or geometric coefficient of variation (q.v.).]

RELATIVE BIAS

Measure of difference between the expected (or mean) value and the true value, expressed as a percentage of the true value.

REPEATABILITY (⟨1225⟩)

The precision within a laboratory over a short interval of time, using the same analyst with the same equipment.

[NOTE—1. ICH Q2(R1) says that repeatability is also termed "intra-assay" precision. In the bioassay context, the better term is *intra-run*, and a "short interval of time" connotes *within-run*. 2. The idea of a "short interval of time" can be problematic with bioassays. If a run requires multiple weeks and consists of a single assay set, then intra-run precision cannot be determined. Alternatively, if a run consists of two assay data sets and a run can be done in a single day, repeatability of the relative potency determination can be assessed.]

REPRODUCIBILITY ⟨1225⟩

The precision between laboratories.

[NOTE—1. Reproducibility includes contributions from repeatability and all factors that contribute to intermediate precision, as well as any additional contributions from inter-laboratory differences. 2. Reproducibility applies to collaborative studies such as those for standardization or portability of methodology. Depending on the design of the collaborative study, it may be possible to separately describe variance components associated with intra- and inter-laboratory sources of variability.]

SPECIFICITY ⟨1225⟩

The ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

[NOTE—1. Typically these components may include impurities, degradants, matrix, etc. See chapter ⟨1225⟩ for further discussion. 2. This definition is also associated with selectivity in other guidances for analytical methods. 3. Specificity can mean the measurement of the specific analyte of interest and no other similar analyte.]

TRUNCATION BIAS

Bias that occurs when some portion of the distribution of responses is not observed or recorded.

[NOTE—1. When there is truncation bias, the distribution of recorded observations does not match the true distribution of responses. 2. Truncation bias may occur in a bioassay that does not report estimates of log potency outside a set potency range. For example, a sample with a true potency at an edge of this range is expected to fail to yield (report) a potency estimate in approximately half of the assays in which it appears. In this example, the mean of the observed potencies will be biased toward log potency 0.]

IV. Terms Related to Validation

DILUTIONAL LINEARITY (ADAPTED FROM ⟨1225⟩)

The ability (within a given range) of a bioassay to obtain measured relative potencies that are directly proportional to the true relative potency of the samples.

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[NOTE— 1. To determine dilutional linearity, sometimes called bioassay analytical linearity, across a range of known relative potency values, analysts examine the relationship between known log potency and mean observed log potency. If that relationship yields an essentially straight line with a *y*-intercept of 0 and a slope of 1, the assay has direct proportionality. If that plot yields an essentially straight line but either the *y*-intercept is not 0 or the slope is not 1, the assay has a proportional linear response. 2. To assess whether the slope is (near) 1.0 requires an a priori equivalence or indifference interval. It is not proper statistical practice to test the null hypothesis that the slope is 1.0 against the alternative that it is not 1.0 and then to conclude a slope of 1.0 if this is not rejected. Bioassay analytical linearity is separate from consideration of the shape of the concentration–response curve. Linearity of concentration–response is not a requirement of bioassay analytical linearity since bioassay analytical linearity is possible regardless of the form of the concentration–response curve. 3. Dilutional linearity is further addressed in ⟨1033⟩.]

QUANTITATION LIMIT (LOWER LIMIT OF QUANTITATION; ADAPTED FROM ⟨1225⟩)

The lowest known relative potency for which the assay has suitable precision and accuracy.

[NOTE—1. This applies to assay results (log potency) rather than the reportable value. 2. The quantitation limit is not commonly determined for relative potency bioassays. Animal assays with serologic endpoints are examples of the use of this term.]

RANGE (ADAPTED FROM ⟨1225⟩)

The interval between the upper and lower known relative potencies (and including those relative potencies) for which the bioassay is demonstrated to have a suitable level of precision, accuracy, and bioassay analytical linearity.

[NOTE—This applies to reportable values (typically a geometric mean) rather than the individual assay results.]

ROBUSTNESS (⟨1225⟩)

A measure of an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters listed in the procedure documentation.

[NOTE—1. Robustness is an indication of a bioassay's reliability during normal usage. For example, a cell culture assay system that is robust to the passage number of the cells can provide potency values with acceptable accuracy and precision across a consistent range of passage numbers. 2. ICH Q2(R1) states:

> The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability [q.v.] parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.[1]]

VALIDATION, ASSAY

Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use.

[NOTE—Formal validations are conducted prospectively according to a written plan that includes justifiable acceptance criteria on validation parameters. See ⟨1033⟩.]

V. Terms Related to Statistical Design and Analysis

ANALYSIS OF VARIANCE (ANOVA)

A statistical tool used to assess contributions to variability from experimental factors.

BLOCKING

The grouping of related experimental units in experimental designs.

[NOTE—1. Blocking often is used to reduce the contribution to variability associated with a factor not of primary interest. 2. Blocks may consist, for example, of groups of animals (a cage, a litter, or a shipment), individual 96-well plates, sections of 96-well plates, or whole 96-well plates grouped by analyst, day, or batches of cells. 3. The goal is to isolate, by statistical design and analysis, a systemic effect, such as cage, so that it does not obscure the comparisons of interest.

> A **complete block design** occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) can be applied to experimental units for that factor within a single block. Note that the two treatment factors *sample* and *concentration* may have different experimental units. For example, if the animals within a cage are all assigned the same concentration but are assigned unique samples, then the experimental unit for concentration is cage and the experimental unit for sample is animal, and cage is a blocking factor for sample.
>
> An **incomplete block design** occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.]

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Confidence Interval

A random interval produced by a statistical method that contains the true (fixed, but unknown) parameter value with a stated confidence level on repeated application of the statistical method.

[Note—See chapter ⟨1010⟩ for more information.]

Crossed (and Partially Crossed)

Two factors are crossed (or fully crossed) if each level of each factor appears with each level of the other factor. Two factors are partially crossed when they are not fully crossed but multiple levels of one factor appear with a common level of the other factor.

[Note—1. For example, in a bioassay in which all samples appear at all dilutions, samples and dilutions are (fully) crossed. In a bioassay validation experiment in which two of four analysts each perform assays on the same set of samples on each of six days and a different pair of analysts is used on each day, the analysts are partially crossed with days. 2. Each factor may be applied to different experimental units, and the factors may be both fully crossed and nested (q.v.), creating a split-unit or split-plot design (q.v.). 3. Experiments with factors that are partially crossed require particular care for proper analysis. 4. A randomized complete block design (q.v.) is a design in which the block factor (which often is treated as a random effect) is crossed with the treatment factor (which usually is treated as a fixed effect).]

Design of Experiments (DOE) [ICH Q8(R2)][5]

A structured, organized method for determining the relationship between factors that affect a process and the output of that process.

[Note—DOE is used in bioassay development and validation; see ⟨1032⟩ and ⟨1033⟩.]

Equivalence Test

A test to demonstrate equivalence (e.g., similarity or conformance to validation acceptance criteria) of two quantities by conformance to an interval acceptance criterion.

[Note—1. An equivalence test differs from most common statistical tests in the nature of the statistical hypotheses. Most common statistical tests are difference tests—that is, the statistical null hypothesis is that of no difference, and the alternative is that there is some difference, without regard to the magnitude or importance of the difference. The difference may be between a characteristic of two populations or between a characteristic of a single population and an accepted value. In equivalence testing the null hypothesis is that the difference is not sufficiently small, and the alternative hypothesis is that the difference is sufficiently small that there is no important difference. In a common statistical difference test one concludes that there is insufficient evidence to establish nonconformance to an acceptance criterion. This may be the result of excess variability and/or an inadequate design. In an equivalence test the conclusion is that the data conform to the acceptance criterion (e.g., slopes are parallel). 2. A common statistical procedure used for equivalence tests is the two one-sided tests (TOST) procedure. 3. The interval acceptance criterion may be one- or two-sided. An example of a one-sided interval is a validation acceptance criterion for a %GCV of not more than XX%]

Expected Mean Square

A mathematical expression of variances estimated by an ANOVA mean square.

Experimental Design

The structure of assigning treatments to experimental units.

[Note—1. Some aspects of experimental design are blocking (q.v.), randomization (q.v.), replication (q.v.), and specific choice of design (cf. ⟨1032⟩). 2. Important components of experimental design include the number of samples, the number of concentrations, and how samples and concentrations are assigned to experimental units and are grouped into blocks. 3. The experimental design influences which statistical methodology should be used to achieve the analytical objective.]

Experimental Unit

The smallest unit to which a distinct level of a treatment is randomly allocated.

[Note—1. Randomization of treatment factors to experimental units is essential in bioassays. 2. Different treatment factors can be applied to different experimental units. For example, samples may be assigned to rows on a 96-well plate, and dilutions may be assigned to columns on the plate. In this case, rows are the experimental units for samples, columns are the experimental units for concentrations, and wells are the experimental units for the interaction of sample and concentration. 3. An experimental unit must be distinguished from a sampling unit, the smallest unit on which a distinct measurement is recorded (e.g., a well). Because the sampling unit is often smaller than the experimental unit, it is an easy mistake to treat sampling units as if they are experimental units. This mistake is called pseudoreplication (q.v.).]

Factor

An assay parameter or operational element that may affect assay response and that varies either within or across assay runs.

[5] ICH. Guidance Q8(R2) Pharmaceutical Development. November 2009. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM073507.pdf. Accessed 27 December 2011.

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[NOTE—In a bioassay there are at least two treatment factors: sample and concentration.

A **fixed factor (fixed effect)** is a factor that is controllable and deliberately set at specific levels in a bioassay. Inference is made to the levels used in the experiment or intermediate values. Sample and concentration are examples of fixed factors in bioassays.

A **random factor (random effect)** is one which is generally not controllable and for which its levels represent a sample of ways in which that factor might vary. In a bioassay, the test organisms, plate, and day are often considered random factors. Whether a factor is treated as random or fixed may depend on the experiment and questions asked.]

FACTORIAL DESIGN

An experimental design in which there are multiple factors and the factors are partially or fully crossed.

In a **full factorial design**, each level of a factor appears with all combinations of levels of all other factors. For example, if factors are reagent batch and incubation time, for a full factorial design all combinations of incubation time and reagent batch must be included.

A **fractional factorial design** is a reduced design in which each level of a factor appears with only a subset of combinations of levels of all other factors and some factor effects (main effects and/or interactions) are deliberately confounded with other combinations of factor effects. Fractional factorial designs should be carefully considered for screening and optimization purposes. This design can be considered without risk of information loss for validation.

GENERAL LINEAR MODEL

A statistical linear model that relates study factors, which can be continuous or discrete, to experimental responses.

INDEPENDENCE

For two measurements or observations *A* and *B* (raw data, assay sets, or relative potencies) to be independent, values for *A* must be unaffected by *B*'s responses and vice versa.

[NOTE—A consequence of the failure to recognize lack of independence is poor characterization of variance. In practice this means that if two potency or relative potency measurements share a common factor that might influence assay outcome (e.g., analyst, cell preparation, incubator, group of animals, or aliquot of Standard samples), then the correct initial assumption is that these relative potency measurements are not independent. The same concern for lack of independence holds if the two potency or relative potency measurements are estimated together from the same model or are in any way associated without including in the model some term that captures the fact that there are two or more potency measurements. As assay experience is gained, an empirical basis may be established (and monitored) so that it is reasonable to treat potency measurements as independent even if the measurements share a common level of a factor. This is the case when it has been demonstrated that a factor does not have a practically significant effect on long-term bioassay results.]

INTERACTION

Two factors are said to interact if the response to one factor depends on the level of the other factor.

LEVEL

A location on the scale of measurement of a factor.

[NOTE—1. Factors have two or more distinct levels. For example, if a bioassay validation experiment employs three values of incubation time and two batches of a key reagent, the levels are the three times for the factor *incubation time* and the two batches for the factor *batch*. 2. Levels of a factor in a bioassay may be quantitative, such as concentration, or categorical, such as sample (i.e., test and reference).]

LOGNORMAL DISTRIBUTION

A distribution of values (assay responses or potencies) where the logarithms of the values have a normal distribution.

[NOTE—1. Most relative potency bioassay measurements are lognormally distributed. 2. The lognormal is a skewed distribution characterized by increased variability with increased level of response.]

MEAN SQUARE

A calculation in ANOVA representing the variability associated with an experimental factor.

MIXED-EFFECTS MODEL

A statistical model that includes both fixed and random effects.

MODELING, STATISTICAL

The mathematical specification of the relationship between inputs (*X*s) and outputs (*Y*s) of a process, e.g., the concentration–response relationship in bioassay or the modeling of the effects of important sources of variation on potency measurement.

[NOTE—1. Modeling includes methods to capture the dependence of the response on the samples, concentration, experimental units, and groups or blocking factors in the assay configuration. 2. Modeling of bioassay data includes making

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many choices, some of which are driven by the assay design and data. For continuous data there is a choice between linear and nonlinear models. For discrete data there is a choice among logit/log models within a larger family of generalized linear models. In limiting dilution assays, published literature advocates Poisson models and Markov chain binomial models. One can use either fixed-effects models or mixed-effects models for bioassay data. On the one hand, the fixed-effects models are more widely available in software and are somewhat less demanding for statisticians to set up. On the other hand, mixed models have advantages over fixed ones: they are more accommodating of missing data and, more importantly, can allow each block to have different slopes, asymptotes, median effective concentrations required to induce a 50% effect (EC_{50}), or relative potencies. Particularly when the analyst is using straight-line models fitted to nonlinear responses or assay systems in which the concentration–response curve varies from block to block, the mixed model captures the behavior of the assay system in a much more realistic and interpretable way. 3. It is essential that any modeling approach for bioassay data should use all available data simultaneously to estimate the variation (or, in a mixed model, each of several sources of variation). It may be necessary to transform the observations before this modeling to include a variance model or to fit a means model (in which there is a predicted effect for each combination of sample and concentration) to get pooled estimate(s) of variation.]

NESTED

A factor *A* is nested within another factor *B* if the levels of *A* are different for every level of *B*.

[NOTE—1. For example, in a bioassay validation experiment two analysts may perform assays on five days each. If the calendar days for the first analyst are distinct from those of the second analyst, days are nested within analyst. 2. Nested factors have a hierarchical relationship. 3. For two factors to be nested they must satisfy the following: (a) they are applied to different-sized experimental units; (b) the larger experimental unit contains more than one of the smaller experimental units; and (c) the factor applied to the smaller experimental unit is not fully crossed (q.v.) with the factor applied to the larger experimental unit. When conditions (a) and (b) are satisfied and the factors are partially crossed, then the experiment is partially crossed and partially nested. Experiments with this structure require particular care for proper analysis.]

PARALLELISM (OF CONCENTRATION–RESPONSE CURVES)

A quality in which the concentration–response curves of the Test sample and the Reference Standard are identical in shape and differ only by a horizontal difference that is a constant function of relative potency.

[NOTE—1. When Test and Reference preparations are similar (q.v.) and assay responses are plotted against log concentrations, the resulting curve for the Test preparation will be the same as that for the Standard but will be shifted horizontally by an amount that is the logarithm of the relative potency. Because of this relationship, similarity (q.v.) is often equated with *parallelism* but they are not the same. See section 3.5, *Slope-Ratio Concentration–Response Models*, in chapter ⟨1034⟩, in which similar samples have concentration–response relationships with a common (or nearly common) *y*-intercept but may differ in their slopes. 2. In practice, it is not possible to demonstrate that the shapes of two curves are identical. Instead, the two curves are shown to be sufficiently algebraically similar (equivalent) in shape. Note that *similar* should be interpreted as "we have evidence that the two curves are close enough in shape" rather than "we do not have evidence that the two curves differ in shape." 3. The assessment of parallelism depends on the type of function used to fit the response curve. Parallelism for a nonlinear assay using a four-parameter logistic fit means that (a) the slopes of the rapidly changing parts of the Test and Reference Standard curves (that is, slope at a tangent to the curve where the first derivative is at a maximum) should be similar; and (b) the upper and lower asymptotes of the response curves (plateaus) should be similar. For straight-line analysis, the slopes of the lines should be similar.]

POINT ESTIMATE

A single-value estimate obtained from statistical calculations.

[NOTE—1. Examples are the average relative bias, the %GCV, and relative potency. 2. The point estimate may be augmented with an *interval estimate* (confidence interval; q.v.) that employs an interval to express the uncertainty in the determination of the point estimate.]

PSEUDOREPLICATION

The misidentification of samples from experimental units as independent and thus true replicates when they actually are not independent.

[NOTE—1. Pseudoreplication results in incorrect inferences because of the incorrect assignment of variability and the appearance of more replicates than are actually present. 2. Lack of recognition of pseudoreplication is critical because it is an easy mistake to make, and the consequences can be serious. For example, pseudoreplicates commonly arise when analysts make a dilution series for each sample in tubes (the dilution series can be made with serial dilutions, single-point dilutions, or any convenient dilution scheme). The analyst then transfers each dilution of each sample to several wells on one or more assay plates. The wells are then pseudoreplicates because they are simply aliquots of a single dilution process and thus are not representative of independent preparations. 3. A simple way to analyze data from pseudoreplicates is to average over the pseudoreplicates (if a transformation of the observed data is used, the transformation should be applied before averaging over pseudoreplicates) before fitting any concentration–response model. In many assay systems, averaging over pseudoreplicates leaves the assay without any replication. A more complex way to use data containing pseudoreplicates is to use a mixed model that treats the pseudoreplicates as a separate random effect. Although pseudoreplication normally is of little value, it can be advantageous when two conditions are satisfied: (a) the pseudoreplicate (e.g., well-to-well) variation is very large compared to the variation associated with replicates; and (b) the cost of pseudoreplicates is much lower than the cost of replicate experimental units.]

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P VALUE (SIGNIFICANCE PROBABILITY)

The probability of observing, in repeated trials, that an experimental outcome is as different or more different than that observed if the null hypothesis is true.

[NOTE—1. *More different* means further from the null hypothesis. 2. Commonly, $P<0.05$ is taken as a threshold for indicating statistically significant differences, although any value for the threshold may be used. Bases for choosing the threshold are the risks (costs) of making a wrong decision; see *type I error* and *type II error*.]

RANDOMIZATION

A process of assignment of treatment to experimental units based on chance so that all equal-sized subgroups of units have an equal chance of receiving a given treatment.

[NOTE—1. The chance mechanism may be an unbiased physical process (rolling unbiased dice, flipping coins, drawing from a well-mixed urn), random-number tables, or computer-generated randomized numbers. Care must be taken in the choice and use of method. Good practice is to use a validated computerized random-number generator. 2. The use of randomization can help to prevent systematic error from becoming associated with particular samples or a dilution pattern and causing bias. For example, in 96-well bioassays, plate effects can be substantial and can cause bias in observed responses or summary measures. In animal studies, a variety of factors associated with individual animals can influence responses. If extraneous factors that influence either plate assays or animal assays are not routinely demonstrated to have been eliminated or minimized so as to be negligible, randomization is essential to obtaining unbiased data required for the calculation of true potency. 3. Randomization is a good practice even when there is evidence that operational factors (e.g., location, time, reagent lot) have little or no effect on the assay system. While randomization may not protect an individual assay (or perhaps a block of an assay) from a (perhaps newly) important operational factor, randomization provides assurance that results from a collection of assays are not biased due to operational factors.]

REPLICATION

A process in which multiple independent experimental units receive the same level of a treatment factor.

[NOTE—1. The purpose of replication is to minimize the effects of uncontrollable sources of random variability. 2. Replication can occur either completely at random or across blocks. Generally, replication within blocks is pseudoreplication (q.v.). 3. Replication of factors that contribute most greatly to variability, or factors that are at the highest levels in a nested layout, usually result in the most effective reduction of random variability.]

TRUE REPLICATES

Samples based on independent experimental units.

STANDARD ERROR OF ESTIMATE

A measure of uncertainty of an estimate of a reportable value or other parameter estimate because of sampling variation.

[NOTE—1. In bioassay the focus is on the precision (standard error) of the relative potency. 2. Standard errors can be made smaller with additional replication. 3. Technically, the standard error of an estimate is the standard deviation of the sampling distribution of the estimate. The term *standard error* is used to distinguish between this usage of standard deviation (that depends on sample size) and the common laboratory usage in which standard deviation (or coefficient of variation) is used to characterize the precision of individual measurements obtained from a procedure. This latter precision does not depend on sample size.]

STATISTICAL PROCESS CONTROL

A set of statistical methods used to monitor shifts and trends in a process.

TYPE I ERROR

The error in statistical hypothesis testing that the alternative hypothesis is accepted when it is false.

[NOTE—The probability of a type I error usually is denoted by α.]

TYPE II ERROR

The error in statistical hypothesis testing that the alternative hypothesis is rejected when it is true.

[NOTE—The probability of a type II error usually is denoted by β.]

VARIANCE COMPONENT ANALYSIS

A statistical analysis that partitions contributions made to total variability by components associated with influential assay factors, e.g., analyst, day, or instrument.

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⟨1031⟩ THE BIOCOMPATIBILITY OF MATERIALS USED IN DRUG CONTAINERS, MEDICAL DEVICES, AND IMPLANTS

Change to read:

This chapter provides guidance on the identification and performance of procedures for evaluating the biocompatibility of drug containers, elastomeric closures, medical devices, and implants. Biocompatibility refers to the tendency of these products to remain biologically inert throughout the duration of their contact with the body. The biocompatibility testing procedures referenced in this chapter are designed to detect the nonspecific, biologically reactive, physical or chemical characteristics of medical products or the materials used in their construction. In combination with chemical assays, these biological procedures can be used to detect and identify the inherent or acquired toxicity of medical products prior to or during their manufacturing and processing.

Preclinical testing procedures to evaluate the safety of the elastomers, plastics, or other polymers used in the construction of medical products are referenced or described in the following general chapters: *Injections and Implanted Drug Products* ⟨1⟩, *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019), *Elastomeric Closures for Injections* ⟨381⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. Specific in vitro and in vivo testing procedures to evaluate the biocompatibility of medical products in patients are described under *Biological Reactivity Tests, In Vitro* ⟨87⟩ and under *Biological Reactivity Tests, In Vivo* ⟨88⟩.

The procedures used to evaluate the biocompatibility of a medical product or its construction materials have been categorized as a panel of biological effects (toxicity procedures): cytotoxicity, sensitization, irritation or intracutaneous reactivity, acute systemic toxicity, subchronic toxicity (repeated), genotoxicity, implantation, hemocompatibility, chronic toxicity (extending beyond 10% of the life span of the test animal or beyond 90 days), carcinogenicity, reproductive or developmental toxicity, and biodegradation.[1] The USP general chapters referring to the toxicity procedures for these categories are indicated in *Table 1*. In addition, pyrogenicity, an area of special toxicity, is evaluated under *Pyrogen Test* ⟨151⟩ and under *Bacterial Endotoxins Test* ⟨85⟩. There are currently no general chapters that detail subchronic toxicity, genotoxicity, chronic toxicity, carcinogenicity, hemotoxicity, reproductive toxicity, or biodegradation testing[2] requirements.

Table 1. Toxicity Procedures in the USP General Chapters

Biological Effect	USP General Chapter
Cytotoxicity	*Biological Reactivity Tests,In Vitro* ⟨87⟩*
Sensitization	*Sensitization Testing* ⟨1184⟩
Irritation or intracutaneous reactivity	*Biological Reactivity Tests, In Vivo* ⟨88⟩†
Systemic toxicity (acute toxicity)	*Biological Reactivity Tests, In Vivo* ⟨88⟩
Implantation	*Biological Reactivity Tests, In Vivo* ⟨88⟩

* Additional general chapters referring to this biological effect include ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019), *Elastomeric Closures for Injections* ⟨381⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩.

† Additional general chapters referring to this biological effect include ⟨1⟩, ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019), *Elastomeric Closures for Injections* ⟨381⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩.

DRUG CONTAINERS

Biocompatibility of Plastic and Other Polymeric Drug Containers

Pharmaceutical containers consist of a container and a closure. Plastic containers may consist of polymers that upon extraction do not alter the stability of the contained product or do not exhibit toxicity. The biocompatibility testing requirements for drug containers are stated under ⟨1⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. As directed in these chapters, the plastic or other polymeric portions of these products are tested according to the procedures set forth under *Biological Reactivity Tests, In Vitro* ⟨87⟩. A plastic or other polymer that does not meet the requirements of *Biological Reactivity Tests, In Vitro* ⟨87⟩ is not a suitable material for a drug container. Materials that meet the in vitro requirements qualify as biocompatible materials without the need for further testing and may be used in the construction of a drug container. If a class designation (classes I–VI) for plastics or other polymers is desired, the appropriate testing procedures are performed as discussed in the section *In Vivo Testing and Class Designation*.

[1] ISO document 10993-1:1997 (or latest version) entitled *Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing.*

[2] See OECD Guidelines for Testing of Chemicals at www.oecd.org.

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Elastomeric Closures

Elastomeric closures are closures that can be pierced by a syringe and maintain their integrity because of their elastic properties. Elastomeric materials may be composed of several chemical entities including fillers, pigments, plasticizers, stabilizers, accelerators, vulcanizing agents, and a natural or a synthetic polymer. These materials are used for manufacturing a product with the desired elastomeric physical properties, and they frequently demonstrate biological reactivity—cellular degeneration and malformation—when tested with in vitro cell cultures.

The biocompatibility of an elastomeric material is evaluated according to the two-stage testing protocol specified in the *Biological Test Procedures* under *Elastomeric Closures for Injections* ⟨381⟩. Unlike plastics or other polymers, an elastomeric material that does not meet the requirements of the first-stage (in vitro) testing may qualify as a biocompatible material by passing the second-stage (in vivo) testing, which consists of the *Systemic Injection Test* and the *Intracutaneous Test* described under *Biological Reactivity Tests, In Vivo* ⟨88⟩. No class or type distinction is made between elastomeric materials that meet the requirements of the first stage of testing and those that qualify as biocompatible materials by meeting the second-stage requirements. Elastomeric materials are not assigned class I–VI designation.

Change to read:

MEDICAL DEVICES AND IMPLANTS

Medical devices and implants, labeled nonpyrogenic, in direct or indirect contact with the cardiovascular system or other soft body tissues, meet the requirements described under ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019). The products listed in this chapter that meet the criteria are solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, dialyzers and dialysis tubing and accessories, transfusion and infusion assemblies, and intramuscular drug delivery catheters. The outlined criteria do not apply to medical products such as orthopedic products, latex gloves, and wound dressings.

The testing requirements described or referenced under ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019) include *Sterility*, *Bacterial endotoxins*, *Pyrogen*, and *Other requirements*. A procedure to evaluate the presence of bacterial endotoxins is set forth under *Bacterial Endotoxins Test* ⟨85⟩, and the limits are set in *Bacterial Endotoxins* under ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019). For devices that cannot be tested by the *Bacterial Endotoxins Test* ⟨85⟩ because of nonremovable inhibition or enhancement, the *Pyrogen Test* ⟨151⟩ is applied. The procedures for evaluating medical devices purported to contain sterile pathways are set forth in *Sterile Devices* under *Sterility Tests* ⟨71⟩. A procedure for evaluating the safety of medical devices is set forth in the *Safety Test* under *Biological Reactivity Tests, In Vivo* ⟨88⟩.

The plastic or other polymer components of medical devices meet the requirements specified for plastics and other polymers under *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩; those made of elastomers meet the requirements under *Elastomeric Closures for Injections* ⟨381⟩. As directed in these chapters, the biocompatibility of the plastic, other polymeric, and elastomeric portions of these products are tested according to the procedures described under *Biological Reactivity Tests, In Vitro* ⟨87⟩.If a class designation for a plastic or other polymer is also required, the appropriate testing procedures described under *Biological Reactivity Tests, In Vivo* ⟨88⟩ are performed.

As required for elastomeric closures, elastomeric materials that do not meet the in vitro requirements may qualify as biocompatible materials and may be used in the construction of medical devices if they meet the requirements of the *Systemic Injection Test* and the *Intracutaneous Test* under *Biological Reactivity Tests, In Vivo* ⟨88⟩. As required for drug containers, plastics and other polymers that do not meet the in vitro testing requirements are not suitable materials for use in medical devices.

IN VITRO TESTING, IN VIVO TESTING, AND CLASS DESIGNATION FOR PLASTICS AND OTHER POLYMERS

The testing requirements specified under *Biological Reactivity Tests, In Vitro* ⟨87⟩ and *Biological Reactivity Tests, In Vivo* ⟨88⟩ are designed to determine the biological reactivity of mammalian cell cultures and the biological response of animals to elastomeric, plastic, and other polymer materials with direct or indirect patient contact. The biological reactivity of these materials may depend on both their surface characteristics and their extractable chemical components. The testing procedures set forth in these chapters can often be performed with the material or an extract of the material under test, unless otherwise specified.

Preparation of Extracts

Evaluation of the biocompatibility of a whole medical product is often not realistic; thus, the use of representative portions or extracts of selected materials may be the only practical alternative for performing the assays. When representative portions of the materials or extracts of the materials under test are used, it is important to consider that raw materials may undergo chemical changes during the manufacturing, processing, and sterilization of a medical product. Although in vitro testing of raw materials can serve as an important screening procedure, a final evaluation of the biocompatibility of a medical product is performed with portions of the finished and sterilized product.

The preparation of extracts is performed according to the procedures set forth under *Biological Reactivity Tests, In Vitro* ⟨87⟩ and under *Biological Reactivity Tests, In Vivo* ⟨88⟩. Extractions may be performed at various temperatures (121°, 70°, 50°, or 37°), for various time intervals (1 hour, 24 hours, or 72 hours), and in different extraction media. The choice of extraction medium for the procedures under *Biological Reactivity Tests, In Vitro* ⟨87⟩ includes *Sodium Chloride Injection* (0.9% NaCl) or tissue culture medium with or without serum. When medium with serum is used, the extraction temperature cannot exceed 37°. In vivo

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extraction medium includes the choices described under *Biological Reactivity Tests, In Vivo* ⟨88⟩ or the solvent to which the drug or medical device is exposed.

When choosing extraction conditions, select the temperature, solvent, and time variables that best mimic the "in use" conditions of the product. The performance of multiple tests at various conditions can be used to simulate variations in the "in use" conditions. Although careful selection of extraction conditions allows the simulation of manufacturing and processing conditions in the testing of raw materials, an evaluation of the biocompatibility of the product is performed with the finished and sterilized product.

In Vitro Testing

The procedures described under *Biological Reactivity Tests, In Vitro* ⟨87⟩ include an *Agar Diffusion Test* (indirect contact test), a *Direct Contact Test*, and an *Elution Test* (extraction test). The sample is biocompatible if the cell cultures do not exhibit greater than a mild reactivity (Grade 2) to the material under test, as described under *Biological Reactivity Tests, In Vitro* ⟨87⟩. The *Agar Diffusion Test* is designed to evaluate the biocompatibility of elastomeric materials. The material is placed on the agar overlay of the cell monolayer, which cushions the cells from physical damage by the material and allows leachable chemicals or materials to diffuse from the elastomer and contact the cell monolayer. Extracts of elastomeric materials are tested by placing the filter paper saturated with an extract of the elastomer on the solidified surface of the agar. The *Direct Contact Test* is designed for elastomeric or plastic materials that will not physically damage cells with which they are in direct contact. Any leachable chemicals diffuse from the material into the serum-supplemented growth medium and directly contact the cell monolayer. The *Elution Test* is designed to evaluate the extracts of polymeric materials. The material may be applied directly to the tissue culture media.

The performance of either the *Agar Diffusion Test* or the *Direct Contact Test* in combination with the *Elution Test* is the preferred testing protocol. Extraction of the product or materials for the *Agar Diffusion Test* or the *Elution Test* is performed as described in the *Preparation of Extracts*.

In Vivo Testing and Class Designation

According to the injection and implantation requirements specified in *Table 1* under *Biological Reactivity Tests, In Vivo* ⟨88⟩, plastics and other polymers are assigned a class designation between class I and class VI. To obtain a plastic or other polymer class designation, extracts of the test material are produced according to the specified procedures in various media.

To evaluate biocompatibility, the extracts are injected systemically and intracutaneously into mice and rabbits or guinea pigs. According to the specified injection requirements, a plastic or other polymer may initially be graded as class I, II, III, or V. If in addition to injection testing, implantation testing using the material itself is performed, the plastic or other polymer may be classified as class IV or class VI.

Change to read:

BIOCOMPATIBILITY OF MEDICAL DEVICES AND IMPLANTS

In addition to evaluating medical products for compendial purposes according to the procedures specified under ⟨1⟩, *Sterility Tests* ⟨71⟩, *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, ▲*Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩▲ (CN 1-May-2019), *Elastomeric Closures for Injections* ⟨381⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, medical devices and implants are evaluated for sensitization, subchronic toxicity, genotoxicity, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation as required by the regulatory agencies.

The guidance provided by the regulatory agencies indicates that the extent of testing that is performed for a medical device or an implant depends on the following factors: (1) the similarity and uniqueness of the product relative to previously marketed ("predicate") products as considered in the *Decision Flowchart*; (2) the extent and duration of the contact between the product and the patient as described in the *Categorization of Medical Devices*; and (3) the material composition of the product as considered in the sections *Decision Flowchart* and *In Vivo Testing and Class Designation*.

Decision Flowchart

Guidance on comparing a medical device or an implant to previously marketed products is provided by the Biocompatibility Decision Flowchart (see *Figure 1*[3]) as adapted from the FDA's Blue Book Memorandum #G95-1. The purpose of the flowchart is to determine whether the available data from previously marketed devices are sufficient to ensure the safety of the device under consideration. As indicated by the flowchart, the material composition and the manufacturing techniques of a product are compared to those of the previously marketed products for the devices that come in direct contact with the body. In addition, the flowchart requires an evaluation of the toxicity of any unique material that has not been used in predicate devices. Responses to the questions posed in the flowchart lead to the conclusion that either the available data are sufficient or additional testing is required to ensure the safety of the product. When additional testing is required, guidance on the identification of appropriate testing procedures is provided in the section *Test Selection Matrix*.

[3] Adapted from the FDA Blue Book Memorandum #G95-1 ("Use of International Standard ISO-10993, 'Biological Evaluation of Medical Devices-Part 1: Evaluation and Testing.'")

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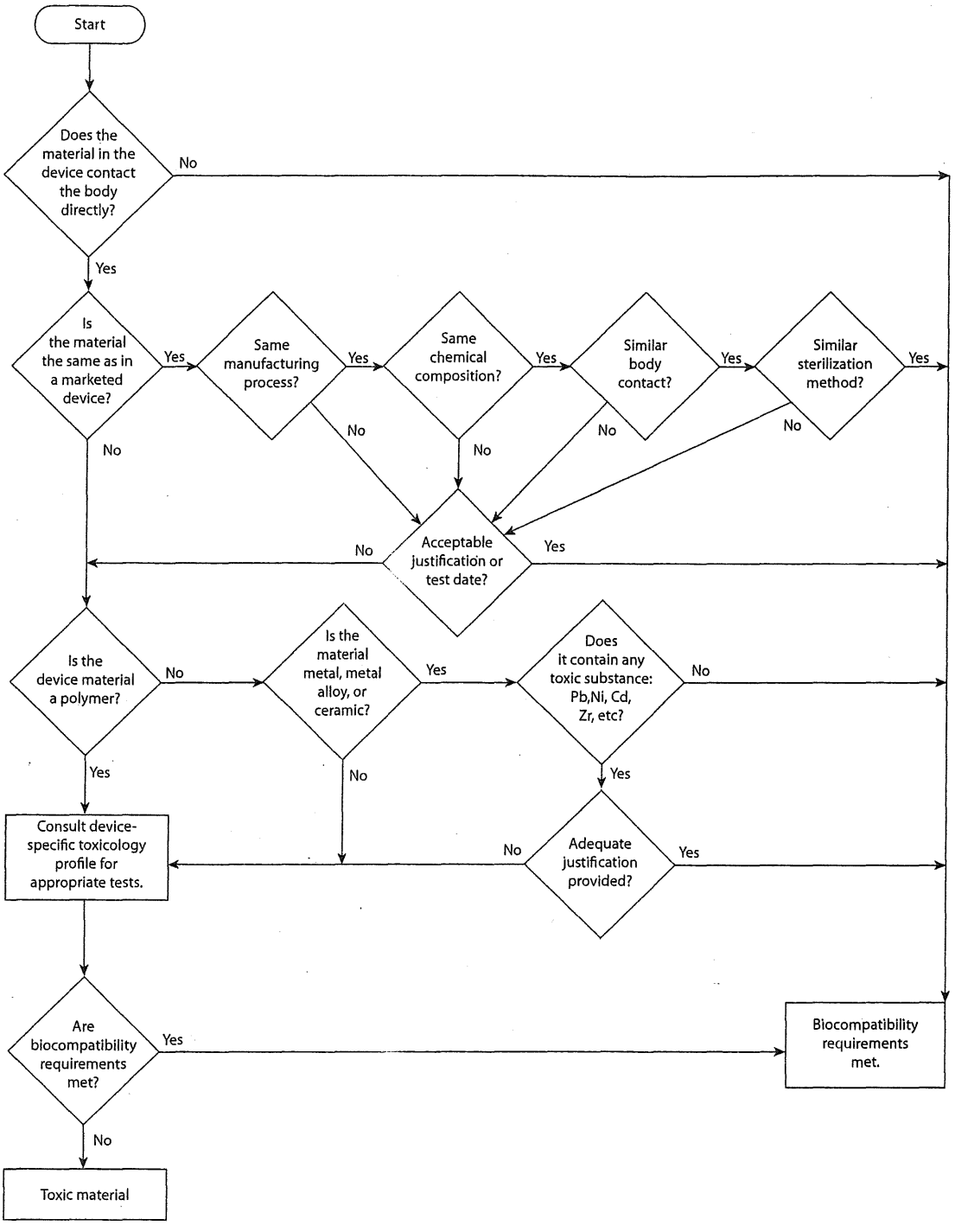

Figure 1. Biocompatibility flowchart.

Categorization of Medical Devices

To facilitate the identification of appropriate testing procedures, medical devices are divided and subdivided, as shown in *Table 2*, according to the nature and extent of their contact with the body. Major categories of medical devices are surface devices, external communicating devices, and implant devices. These are then further subcategorized. Some examples of medical devices and implants belonging to each of the subcategories are also presented in *Table 2*.

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Table 2. Classification and Examples of Medical Devices

Device Category	Device Subcategory	Nature or Extent of Contact	Some Examples
Surface Devices	Skin	Devices that contact intact skin surfaces only	Electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types
	Mucosal Membrane	Devices communicating with intact mucosal membranes	Contact lenses, urinary catheters, intravaginal and intraintestinal devices (stomach tubes, sigmoidoscopes, colonoscopes, gas troscopes), endotracheal tubes, bronchoscopes, dental prostheses, orthodontic devices, and intrauterine devices
	Breached or Compromised Surfaces	Devices that contact breached or otherwise compromised body surfaces	Ulcer, burn, and granulation tissue dressings or healing devices and occlusive patches
External Communicating Devices	Blood Path, Indirect	Devices that contact the blood path at one point and serve as a conduit for entry into the vascular system	Solution administration sets, extension sets, transfer sets, and blood administration sets
	Tissue, Bone, or Dentin Communicating	Devices and materials communicating with tissue, bone, or pulp and dentin system	Laparoscopes, arthroscopes, draining systems, dental cements, dental filling materials, and skin staples
	Circulating blood	Devices that contact circulating blood	Intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporeal oxygenator tubing and accessories, dialyzers, dialysis tubing and accessories, hemoadsorbents, and immunoadsorbents
Implant Devices	Tissue or Bone	Devices principally contacting bone or principally contacting tissue and tissue fluid	Examples of the former are orthopedic pins, plates, replacement joints, bone prostheses, cements, and intraosseous devices; examples of the latter are pacemakers, drug supply devices, neuromuscular sensors and simulators, replacement tendons, breast implants, artificial larynxes, subperiosteal implants, and ligation clips
	Blood	Devices principally contacting blood	Pacemaker electrodes, artificial arteriovenous fistulae, heart valves, vascular grafts, internal drug delivery catheters, and ventricular-assist devices

Test Selection Matrix

The matrix provides guidance on the identification of appropriate biological testing procedures for the three categories of medical devices: tests for *Surface Devices* (see *Table 3*), tests for *External Communicating Devices* (see *Table 4*), and tests for *Implant Devices* (see *Table 5*). Each category of devices is subcategorized and then even further subdivided according to the duration of the contact between the device and the body. The duration of contact is defined as (A) limited (less than 24 hours); (B) prolonged (24 hours to 30 days); or (C) permanent (more than 30 days). The biological effects that are included in the matrix are cytotoxicity, sensitization, irritation or intracutaneous reactivity, systemic toxicity, subchronic toxicity, genotoxicity, implantation, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation. The general chapters that contain toxicity testing procedures for these biological effects are indicated in *Table 1*.

Each subcategory in the matrix has an associated panel of testing requirements. Generally, the number of tests in the panel increases as the duration of the contact between the device and the body is extended and as the device or implant comes in closer contact with the circulatory system. Within several subcategories, the option of performing additional tests beyond those required should be considered on a case-by-case basis. Specific situations such as use of permanent implant devices or external communicating devices for pregnant women and children have to be taken into consideration in the manufacturer's decision to include reproductive or developmental testing. Guidance on the identification of possible additional testing procedures is provided in the matrix for each subcategory of medical devices.

GUIDANCE IN SELECTING THE PLASTIC OR OTHER POLYMER CLASS DESIGNATION FOR A MEDICAL DEVICE

To provide guidance on selecting the appropriate plastic or other polymer class designation for a medical device, each subcategory of *Surface Devices* (see *Figure 2*)

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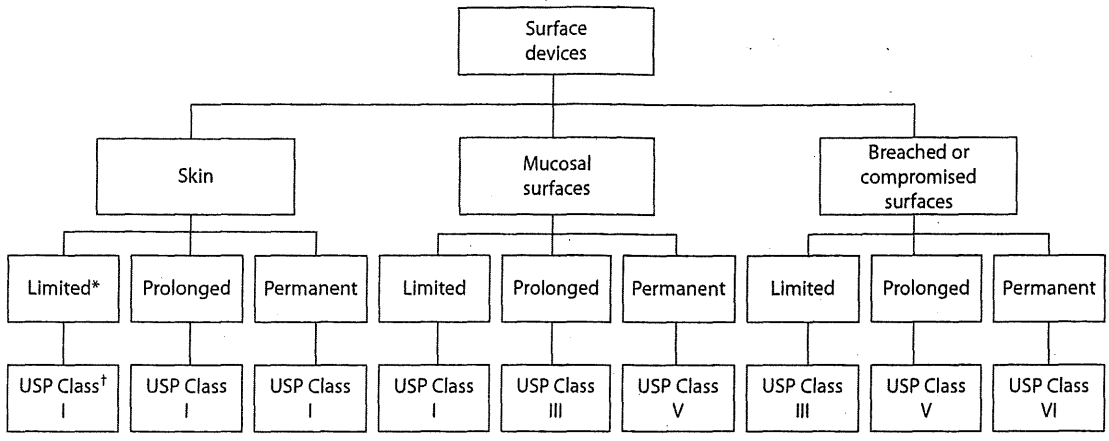

Figure 2. USP plastic and other polymer class requirements for surface devices.*Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days. †USP Plastic Class designation.

and *External Communicating Devices* (see *Figure 3*)

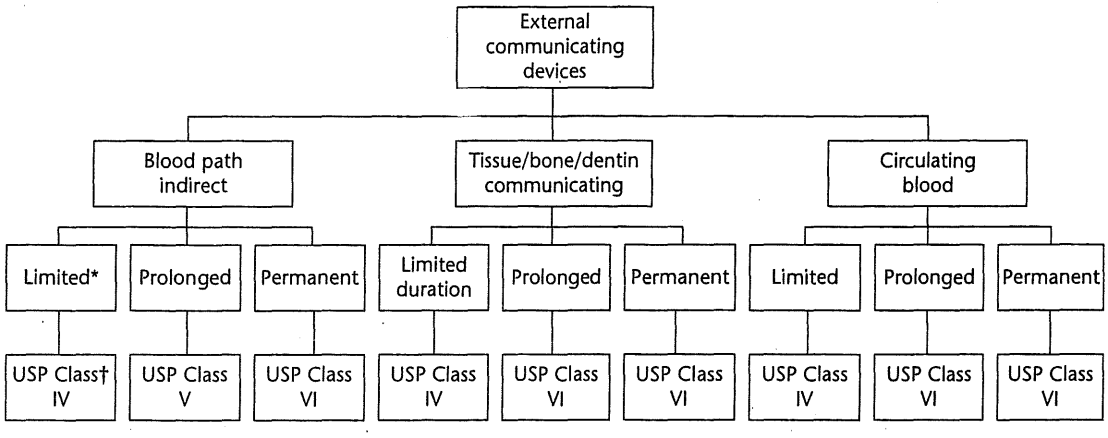

Figure 3. USP plastic and other polymer class requirements for external communicating devices. *Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days.†USP Plastic Class designation.

is assigned a USP Plastic Class designation (see *Biological Reactivity Tests, In Vivo* ⟨88⟩). If the tests for each USP class designation are not sufficient for a specific device, the manufacturer or the practitioner must develop an appropriate set of tests. The indicated numerical class number increases relative to the duration (risk) of contact between the device and the body. In the category of *Implant Devices,* the exclusive use of class VI is mandatory. The assignment of USP Plastic Class designation is based on the test selection matrices illustrated in *Tables 3*, *4*, and *5*.

The assignment of a plastic or other polymer class designation to a subcategory is not intended to restrict the use of higher classes of plastics or other polymers. Although the assigned class defines the lowest numerical class of plastic or other polymer that may be used in the corresponding device, the use of a numerically higher class of plastic is optional. When a device can be defined as belonging to more than one device category, the plastic or other polymer should meet the requirements of the highest numerical class.

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Table 3. Test Selection Matrix for Surface Devices*

Device Categories		Biological Effect[b]											
Body Contact		Contact Duration[a]	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity	Genotoxicity	Implantation	Hemocompatability	Chronic Toxicity	Carcinogenicity	Reproductive or Development Toxicity	Biodegradation
Surface Devices	Skin	A	X	X	X	—	—	—	—	—	—	—	—	—
		B	X	X	X	—	—	—	—	—	—	—	—	—
		C	X	X	X	—	—	—	—	—	—	—	—	—
	Mucosal Membrane	A	X	X	X	—	—	—	—	—	—	—	—	—
		B	X	X	X	O	O	—	O	—	—	—	—	—
		C	X	X	X	O	X	X	O	—	O	—	—	—
	Breached or Compromised Surfaces	A	X	X	X	O	—	—	—	—	—	—	—	—
		B	X	X	X	O	O	—	O	—	—	—	—	—
		C	X	X	X	O	X	X	O	—	O	—	—	—

* Adapted from the FDA's Blue Book Memorandum #G95-1 (*Table 1. Initial Evaluation Tests for Consideration* and *Table 2. Supplementary Evaluation Tests for Consideration*).
[a] Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).
[b] Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

Table 4. Test Selection Matrix for External Communicating Devices*

Device Categories		Biological Effect[b]											
Body Contact		Contact Duration[a]	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity	Genotoxicity	Implantation	Hemocompatability	Chronic Toxicity	Carcinogenicity	Reproductive or Development Toxicity	Biodegradation
External Communicating Devices	Blood Path, Indirect	A	X	X	X	X	—	—	—	X	—	—	—	—
		B	X	X	X	X	O	—	—	X	—	—	—	—
		C	X	X	O	X	X	X	O	X	X	X	—	—
	Tissue, Bone, or Dentin Communicating	A	X	X	X	O	—	—	—	—	—	—	—	—
		B	X	X	O	O	O	X	X	—	—	—	—	—
		C	X	X	O	O	O	X	X	—	X	X	—	—
	Circulating Blood	A	X	X	X	X	—	O	—	X	—	—	—	—
		B	X	X	X	X	O	X	O	X	—	—	—	—
		C	X	X	X	X	X	X	O	X	X	X	—	—

* Adapted from the FDA's Blue Book Memorandum #G95-1 (*Table 1. Initial Evaluation Tests for Consideration* and *Table 2. Supplementary Evaluation Tests for Consideration*).
[a] Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).
[b] Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

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Table 5. Test Selection Matrix for Implant Devices*

Device Categories			Biological Effect[b]											
Body Contact		Contact Duration[a]	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity	Genotoxicity	Implantation	Hemocompatability	Chronic Toxicity	Carcinogenicity	Reproductive or Developmental Toxicity	Biodegradation
Implant Devices	Tissue or Bone	A	X	X	X	O	—	—	—	—	—	—	—	—
		B	X	X	O	O	O	X	X	—	—	—	—	—
		C	X	X	O	O	O	X	X	—	X	X	—	—
	Blood	A	X	X	X	X	—	—	X	X	—	—	—	—
		B	X	X	X	X	O	X	X	X	—	—	—	—
		C	X	X	X	X	X	X	X	X	X	X	—	—

* Adapted from the FDA's Blue Book Memorandum #G95-1 (*Table 1. Initial Evaluation Tests for Consideration* and *Table 2. Supplementary Evaluation Tests for Consideration*).

[a] Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).

[b] Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

⟨1032⟩ DESIGN AND DEVELOPMENT OF BIOLOGICAL ASSAYS

1. INTRODUCTION

1.1 Purpose and Scope

General chapter *Design and Development of Biological Assays* ⟨1032⟩ presents methodology for the development of bioassay procedures that have sound experimental design, that provide data that can be analyzed using well-founded statistical principles, and that are fit for their specific use.

General chapter ⟨1032⟩ is one of a group of five general chapters that focus on relative potency assays, in which the activity of a Test material is quantified by comparison to the activity of a Standard material. However, many of the principles can be applied to other assay systems.

This general chapter is intended to guide the design and development of a bioassay for a drug substance or product intended for commercial distribution. Although adoption of this chapter's recommended methods may be resource intensive during assay development, early implementation can yield benefits. Lastly, the perspectives and methods described herein are those recommended from among the many alternatives which contemporary bioassay theory and practice offers.

FOCUS ON RELATIVE POTENCY

Because of the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically *similar*, *statistical similarity* (a consequence of the Test and Standard similarity) should be present, and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unitless measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations. For the purpose of the relative comparison of Test to Standard, the potency of the Standard is usually assigned a value of 1 (or 100%). The Standard can be a material established as such by a national (e.g., USP) or international (e.g., WHO) organization, or it could be an internal Standard.

1.2 Audience

This chapter is intended for both the practicing bioassay analyst and the statistician who are engaged in developing a bioassay. The former will find guidance for implementing bioassay structure and methodology to achieve analytical goals while reliably demonstrating the biological activity of interest, and the latter will gain insights regarding the constraints of biology that can prove challenging to balance with a rigorous practice of statistics.

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2. BIOASSAY FITNESS FOR USE

To evaluate whether an assay is fit for use, analysts must specify clearly the purpose(s) for performing the bioassay. Common uses for a bioassay include lot release of drug substance (active pharmaceutical ingredient) and drug product; assessment of stability; qualification of Standard and other critical reagents; characterization of process intermediates and formulations; characterization of contaminants and degradation products; and support of changes in the product production process. The relative accuracy, specificity, precision, and robustness requirements may be different for each of these potential uses. It is a good strategy to develop and validate a bioassay to support multiple intended uses; for example, a bioassay primarily developed for batch release may serve other purposes. Decisions about fitness for use are based on scientific and statistical considerations, as well as practical considerations such as cost, turnaround time, and throughput requirements for the assay.

When assays are used for lot release, a linear-model bioassay may allow sufficient assessment of similarity. For bioassays used to support stability, comparability, to qualify reference materials or critical reagents, or in association with changes in the production or assay processes, it is generally useful to assess similarity using the entire concentration–response curve, including the asymptotes (if present).

2.1 Process Development

Bioassays are generally required in the development and optimization of product manufacturing, including formulation and scale-up processes. Bioassays can be used to evaluate purification strategies, optimize product yield, and measure product stability. Because samples taken throughout the process are often analyzed and compared, sample matrix effects that may affect assay response should be carefully studied to determine an assay's fitness for use. For relative potency measures, the Standard material may require dilution into a suitable matrix for quantitation. The bioassay's precision and accuracy should be sufficient for measuring process performance or for assessing and comparing the stability of candidate formulations.

2.2 Process Characterization

Bioassays may be performed to assess the effect on drug potency associated with different stages of drug manufacture or with changes in the manufacturing process (e.g., to demonstrate product equivalence before and after process changes are made). Bioassays used in this type of application may be qualitative or quantitative.

2.3 Product Release

Bioassays are used to evaluate the potency of the drug before commercial product release. To the extent possible, the assay should reflect or mimic the product's known or intended mechanism of action. If the bioassay does not include the functional biology directly associated with the mechanism of action, it may be necessary to demonstrate a relationship between the bioassay's estimated potency determinations and those of some other assay that better or otherwise reflects putative functional activity.

For product-release testing, product specifications are established to define a minimum or range of potency values that are acceptable for product. The precision of the reportable value from the bioassay must support the number of significant digits listed in the specification (see general chapter *Biological Assay Validation* ⟨1033⟩), and, in conjunction with relative accuracy, support the specification range. In order to meet these specifications, manufacturing quality control will have sufficiently narrow product release specifications in order to accommodate any loss of activity due to instability and uncertainty in the release assay.

2.4 Process Intermediates

Bioassay assessment of process intermediates can provide information regarding specificity. Formulation and fill strategies may rely on bioassays in order to ensure that drug product, including that in final container, will meet its established specifications. For example, unformulated bulk materials may be held and evaluated for potency. Bulks may be pooled with other bulk lots, diluted, or reworked based on the potency results. For these types of applications, the bioassay must be capable of measuring product activity in different matrices. In some cases, a separate Standard material is made and is used to calculate relative potency for the process intermediate.

2.5 Stability

The potency assay may be used to assess biotechnology and vaccine product stability. Information from stability studies, performed during development under actual and/or accelerated or stressed storage conditions, may be used to establish shelf life duration as well as to identify and estimate degradation products and degradation rates. Post licensure stability studies may be used to monitor product stability. Knowledge of both short-term and long-term variability of the bioassay is important to assure an acceptable level of uncertainty in potency measures obtained.

2.6 Qualification of Reagents

The quantitative characterization of a new Standard requires an accurate and precise measurement of the new Standard's biological activity. This measurement is used either to establish that the new Standard lot is equivalent to the previous lot or to assign it a label potency to which Test samples can be compared. Additional replication (beyond routine testing) may be required to achieve greater precision in the potency measurement of the new Standard material. Additionally, the bioassay may

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be used to qualify a cell culture reagent such as fetal bovine serum. The fitness for use in such cases is tied to the ability of the assay to screen reagent lots and to ensure that lots that may bias or compromise the relative potency measurements are not accepted.

2.7 Product Integrity

Biotechnology, biological, and vaccine products may contain a population of heterogeneous material, including the intended predominant product material. Some process impurities and degradation products may be active, partially active, inactive in, or antagonistic to, the response measured in the bioassay. For product variants or derivatives for which changes in structure or relative composition may be associated with subtle yet characteristic changes in the bioassay response (e.g., change in slope or asymptote), the bioassay may be useful in the detection and measurement of these variants or derivatives. Studies that identify characteristic changes associated with variants of the intended product help ensure consistent product performance. Whenever practical, the bioassay should be accompanied by orthogonal methods that are sensitive to product variants, process impurities, and/or degradation products.

3. BIOASSAY FUNDAMENTALS

3.1 In Vivo Bioassays

In vivo potency assays are bioassays in which sets of dilutions of the Standard and Test materials are administered to animals and the concentration–response relationships are used to estimate potency. For some animal assays, the endpoint is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoeitin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has substantially diminished. Cost, low throughput, ethical, and other practical issues argue against the use of animal bioassays. Regulatory agencies have encouraged the responsible limitation of animal use whenever possible (see The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities; February 2004). When in vitro activity is not strongly associated with in vivo activity (e.g., EPO), the combination of an in vitro cell-based assay and a suitable physicochemical method (e.g., IEF, glycan analysis) may substitute for in vivo assays. However, a need for in vivo assays may remain when in vitro assays cannot detect differences that are critical in regard to a drug's intended biological function.

Animals' physiological responses to biological drugs (including vaccines) may predict patients' responses. Selection of animal test subjects by species, strain, gender, and maturity or weight range is guided by the goal of developing a representative and sensitive model with which to assess the activity of Test samples.

Some assay methods lend themselves to the use of colony versus naive animals. For example, pyrogen and insulin testing benefit from using experienced colony rabbits that provide a reliable response capacity. If animals recently introduced to the colony fail to respond as expected after several administrations of a compound, they should be culled from the colony so they do not cause future invalid or indeterminate assay results. In the case of assaying highly antigenic compounds for pyrogens, however, naive animals should be used to avoid generating inaccurate or confounded results. Other colony advantages include common controlled environmental conditions (macro/room, and micro/rack), consistent feeding schedule, provision of water, and husbandry routine.

Historical data including colony records and assay data can be used to identify factors that influence assay performance. The influence of biasing factors can be reduced by applying randomization principles such as distribution of weight ranges across dose groups, group assignments from shipping containers to different cages, or use of computer-generated or deck patterns for injection/dosing. A test animal must be healthy and have time to stabilize in its environment to be suitable for use in a bioassay. Factors that combine to influence an animal's state of health include proper nutrition, hydration, freedom from physical and psychological stressors, adequate housing sanitization, controlled light cycle (diurnal/nocturnal), experienced handling, skillful injections and bleedings, and absence of noise or vibration. Daily observation of test animals is essential for maintenance of health, and veterinary care must be available to evaluate issues that have the potential to compromise the validity of bioassay results.

3.2 Ex Vivo Bioassays

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test sample. In the case of cytokines, the majority of assays use cells from the hematopoietic system or subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells.

Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of in vivo assays to minimize assay variability and bias. The level of effort to manage bias (e.g., via randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

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3.3 In Vitro (Cell-Based) Bioassays

Bioassays using cell lines that respond to specific ligands or infectious agents can be used for lot-release assays. These cell lines can be derived from tumors, immortalized as factor-dependent cell lines, or engineered cell lines transfected with appropriate receptors. Additionally, nontransformed cell lines which can be maintained over a sufficient number of passages (e.g., fibroblasts) may also be used. Regardless of cell line, there is an expectation of adequately equivalent potency response through some number of continuous passages. Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response, stable expression of receptors and signaling mechanisms, and longer stability. The cellular responses to the protein of interest depend on the drug's MOA and the duration of exposure. Such responses include cell proliferation, cell killing, antiviral activity, differentiation, cytokine/mediator secretion, and enzyme activation. Assays involving these responses may require incubation of the cells over several days, during which time contamination, uneven evaporation, or other location effects may arise. Comparatively rapid responses based on an intracellular signaling mechanism—such as second messengers, protein kinase activation, or reporter gene expression—have proven acceptable to regulatory authorities. Lastly, most cell lines used for bioassays express receptors for multiple cytokines and growth factors. This lack of specificity may not be detrimental if the Test sample's specificity is demonstrated.

Cell-based bioassay design should reflect knowledge of the factors that influence the response of the cells to the active analyte. Response variability is often reflected in parameters such as slope, EC_{50} of the concentration–response curve, or the response range (maximum minus minimum response). Even though relative potency methodology minimizes the effects on potency estimates of variation in these parameters among assays, and among blocks within an assay, such response variability can make an assay difficult to manage (i.e., it may be difficult to assess system suitability). Hence, while assay development should be focused primarily on the properties of potency, efforts to identify and control variation in the concentration–response relationship are also appropriate. For blocked assays (e.g., multiple cell culture plates in an assay) with appreciable variation in curve shape among blocks, an analysis that does not properly include blocks will yield inflated estimates of within-assay variation, making similarity assessment particularly difficult. Two strategies are available for addressing variation among blocks: one, a laboratory effort to identify and control sources of variation and two, a statistical effort to build and use a blocked design and analysis. Combining these strategies can be particularly effective.

The development of a cell-based bioassay begins with the selection or generation of a cell line. An important first step when developing a cell-based assay to assess a commercial product is to verify that the cell line of interest is not restricted to research use only. To ensure an adequate and consistent supply of cells for product testing, a cell bank should be generated if possible. To the extent possible, information regarding functional and genetic characteristics of the bioassay's cell line should be documented, including details of the cell line's history from origin to banking. For example, for a recombinant cell line this might include the identification of the source of the parental cell line (internal cell bank, external repository, etc.), of the DNA sequences used for transfection, and of the subsequent selection and functional testing regimen that resulted in selection of the cell line. Ideally, though not always practical, sufficient information is available to permit recreation of a similar cell line if necessary. Pertinent information may include identity (e.g., isoenzyme, phenotypic markers, genetic analysis); morphology (e.g., archived photographic images); purity (e.g., mycoplasma, bacteria, fungus and virus testing); cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions); thaw viability (immediately after being frozen and after time in storage); growth characteristics (e.g., cell doubling times); and functional stability (e.g., ploidy).

Cell characterization and vigilance regarding aspects of assay performance that reflect on cell status are necessary to ensure the quality and longevity of cell banks for use in the QC environment. The general health and metabolic state of the cells at the time of bioassay can substantially influence the test results. After a cell line has been characterized and is ready for banking, analysts typically prepare a two-tiered bank (Master and Working). A Master Cell Bank is created as the source for the Working Cell Bank. The Working Cell Bank is derived by expansion of one or more vials of the Master Cell Bank. The size of the banks depends on the growth characteristics of the cells, the number of cells required for each assay, and how often the assay will be performed. Some cells may be sensitive to cryopreservation, thawing, and culture conditions, and the banks must be carefully prepared and characterized before being used for validation studies and for regular use in the QC laboratory.

There follow factors that may affect bioassay response and the assessment of potency, that are common to many cell-based bioassays: cell type (adherent or nonadherent); cell thawing; plating density (at thaw and during seed train maintenance) and confluence (adherent cells); culture vessels; growth, staging, and assay media; serum requirements (source, heat inactivation, gamma irradiation); incubation conditions (temperature, CO_2, humidity, culture times from thaw); cell harvesting reagents and techniques (for adherent cells, method of dissociation); cell sorting; cell counting; determination of cell health (growth rate, viability, yield); cell passage number and passaging schedule; cell line stability (genetic, receptor, marker, gene expression level); and starvation or stimulation steps. This list is not exhaustive, and analysts with comprehensive understanding and experience with the cell line should be involved during assay development. These experienced individuals should identify factors that might influence assay outcomes and establish strategies for an appropriate level of control whenever possible.

3.4 Standard

The Standard is a critical reagent in bioassays because of the necessity to have a reliable material to which a Test preparation can be quantitatively compared. The Standard may be assigned a unitage or specific activity that represents fully (100%) potent material. Where possible, a Standard should be representative of the samples to be tested in the bioassay. Testing performed to qualify a Standard may be more rigorous than the routine testing used for lot release.

A Standard must be stored under conditions that preserve its full potency for the intended duration of its use. To this end, the Standard may be stored under conditions that are different from the normal storage of the drug substance or drug product. These could include a different temperature (e.g., −70° or −20°, instead of 2°–8°), a different container (e.g., plastic vials instead of syringes), a different formulation (e.g., lyophilizable formulation or the addition of carrier proteins such as human serum

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albumin, stabilizers, etc.). The Standard material should be tested for stability at appropriate intervals. System suitability criteria of the bioassay such as maximum or background response, EC_{50} slope, or potency of assay control may be used to detect change in the activity of the Standard. Accelerated stability studies can be performed to estimate degradation rates and establish recognizable characteristics of Standard instability.

At later stages in clinical development, the Standard may be prepared using the manufacturing process employed in pivotal clinical trials. If the Standard formulation is different from that used in the drug product process, it is important to demonstrate that the assay's assessment of similarity and estimate of potency is not sensitive to the differences in formulation. An initial Standard may be referred to as the *Primary Standard*. Subsequent Standards can be prepared using current manufacturing processes and can be designated *Working Standards*. Separate SOPs may be required for establishing these standards for each product. Bias in potency measurements sometimes can arise if the activity of the Standard gradually changes. Also, loss of similarity may be observed if, with time, the Standard undergoes changes in glycosylation. It is prudent to archive aliquots of each Standard lot for assessment of comparability with later Standards and for the investigation of assay drift.

4. STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS

The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample.

4.1 Data

Fundamentally, there are two bioassay data types: quantitative and quantal (categorical). Quantitative data can be either continuous (not limited to discrete observations; e.g., collected from an instrument), count (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titers). Quantal data are often dichotomous; for example, life/death in an animal response model or positivity/negativity in a plate-based infectivity assay that results in destruction of a cell monolayer following administration of an infectious agent. Quantitative data can be transformed to quantal data by selecting a threshold that distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, as by adding (or subtracting) a measure of uncertainty (such as two or three times the standard deviation of negative control responses) to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information.

4.2 Assumptions

A key assumption for the analysis of most bioassays is that the Standard and Test samples contain the same effective analyte or population of analytes and thus may be expected to behave similarly in the bioassay. This is termed *similarity*. As will be shown in more detail in the general chapter *Analysis of Biological Assays* ⟨1034⟩ for specific statistical models, biological similarity implies that statistical similarity is present (for parallel-line and parallel-curve models, the Standard and Test curves are parallel; for slope-ratio models, the Standard and Test lines have a common intercept). The reverse is not true. Statistical similarity (parallel lines, parallel curves, or common intercept, as appropriate) does not ensure biological similarity. However, failure to satisfy statistical similarity may be taken as evidence against biological similarity. The existence of a Standard–Test sample pair that passes the assessment of statistical similarity is thus a necessary but not sufficient condition for the satisfaction of the key assumption of biological similarity. Biological similarity thus remains, unavoidably, an assumption. Departures from statistical similarity that are consistent in value across replicate assays may be indicative of matrix effects or of real differences between Test and Standard materials. This is true even if the departure from statistical similarity is sufficiently small to support determination of a relative potency.

In many assays multiple compounds will yield similar concentration–response curves. It may be reasonable to use a biological assay system to describe or even compare response curves from different compounds. But it is not appropriate to report relative potency unless the Standard and Test samples contain only the same active analyte or population of analytes. Biological products typically exhibit lot-to-lot variation in the distribution of analytes (i.e., most biological products contain an intended product and, at acceptably low levels, some process contaminants that may be active in the bioassay). Assessment of similarity is then, at least partially, an assessment of whether the distribution of analytes in the Test sample is close enough to that of the distribution in the Standard sample for relative potency to be meaningful; that is, the assay is a comparison of like to like. When there is evidence (from methods other than the bioassay) that the Standard and Test samples do not contain the same active compound(s), the assumption of biological similarity is not satisfied, and it is not appropriate to report relative potency.

Other common statistical assumptions in the analysis of quantitative bioassays are constant variance of the responses around the fitted model (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation* for further discussion), normally distributed residuals (a residual is the difference between an observed response and the response predicted by the model), and independence of the residuals.

Constant variance, normality, and independence are interrelated in the practice of bioassay. For bioassays with a quantitative response, a well-chosen data transformation may be used to obtain approximately constant variance and a nearly normal distribution of residuals. Once such transformation has been imposed, the remaining assumption of independence then remains to be addressed via reflection of the assay design structure in the analysis model. Independence of residuals is important for assessing system and sample suitability.

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4.3 Variance Heterogeneity, Weighting, and Transformation

Simple analysis of quantitative bioassay data requires that the data be approximately normally distributed with near constant variance across the range of the data. For linear and nonlinear regression models, the variance referred to here is the residual variance from the fit of the model. Constant variance is often not observed; *variance heterogeneity* may manifest as an increase in variability with increase in response. If the variances are not equal but the data are analyzed as though they are, the estimate of relative potency may still be reasonable; however, failure to address nonconstant variance around the fitted concentration–response model results in an unreliable estimate of within-assay variance. Further, the assessment of statistical similarity may not be accurate, and standard errors and confidence intervals for all parameters (including a Fieller's Theorem-based interval for the relative potency) should not be used. Confidence intervals for relative potency that combine potency estimates from multiple assays may be erroneous if within-assay error is used for confidence interval calculation.

Constancy of variance may be assessed by means of residual plots, Box-Cox (or power law) analysis, or Levene's test. With Levene's test, rather than relying on the p value, change in the statistic obtained is useful as a basis for judging whether homogeneity is improved or worsened. Variance is best assessed on a large body of assay data. Using only the variance among replicates from the current assay is not appropriate, because there are too few data to properly determine truly representative variances specific to each concentration. Data on variance is sparse during development; it is prudent to re-assess variance during validation and to monitor it periodically during ongoing use of the assay.

Two methods used to mitigate variance heterogeneity are transformation and weighting. Lack of constant variance can be addressed with a suitable transformation. Additionally, transformation can improve the normality of residuals and the fit of some statistical models to the data. A transformation should be chosen for an assay system during development, checked during validation, used consistently in routine assay practice, and checked periodically. Bioassay data are commonly displayed with log-transformed concentration; slope-ratio assays are displayed with concentration on the original scale.

Transformation may be performed to the response data as well as to the concentration data. Common choices for a transformation of the response include log, square root (for counts), reciprocal, and, for count data with known asymptotes, logit of the percent of maximum response. Log transformations are commonly used, as they may make nearly linear a useful segment of the concentration–response relationship, and because of the ease of transforming back to the original scale for interpretation. A log–log fit may be performed on data exhibiting nonlinear behavior. Other alternatives are available; i.e., data may be transformed by the inverse of the *Power of the Mean* (POM) function. A POM coefficient of k = 2 corresponds to a log transformation of the data. For further discussion of relationships between log-transformed and untransformed data, see *Appendix* in the general chapter *Biological Assay Validation* ⟨1033⟩.

Note that transformation of the data requires re-evaluation of the model used to fit the data. From a statistical perspective there is nothing special about the original scale of measurement; any transformation that improves accordance with assumptions is acceptable. Analysts should recognize, however, that transformations, choice of statistical model, and choice of weighting scheme are interrelated. If a transformation is used, that may affect the choice of model. That is, transforming the response by a log or square root, for example, may change the shape of the response curve, and, for a linear model, may change the range of concentrations for which the responses are nearly straight and nearly parallel.

For assays with non-constant variance, a weighted analysis may be a reasonable option. Though weighting cannot address lack of residual normality, it is a valid statistical approach to placing emphasis on more precise data. Ideally, weights may be based on the inverse of the predicted within-assay (or within-block) variance of each response where the predictors of variance are independent of responses observed in a specific assay.

In practice, many bioassays have relatively large variation in log EC_{50} (compared to the variation in log relative potency) among assays (and sometimes among blocks within assay). If not addressed in the variance model, this variation in log EC_{50} induces what appears to be large variation in response near the mean log EC_{50}, often yielding too-low weights for observations near the EC_{50}.

If the assay is fairly stable (low variability in EC_{50}), an alternative is to look at variance as a function of concentration. While not ideal, an approach using concentration-dependent variances may be reasonable when the weights are estimated from a large number of assays, the variances are small, any imbalance in the number of observations across concentrations is addressed in the variance model, and there are no unusual observations (outliers). This possibility can be examined by plotting the response variance at each concentration (preferably pooled across multiple assays) against concentration and then against a function of concentration (e.g., concentration squared). Variance will be proportional to the function of concentration where this plot approximates a straight line. The apparent slope of this line is informative, in that a horizontal line indicates no weighting is needed. If a function that yields a linear plot can be found, then the weights are taken as proportional to the reciprocal of that function. There may be no such function, particularly if the variation is higher (or lower) at both extremes of the concentration range studied.

Whether a model or historical data are used, the goal is to capture the relative variability at each concentration. It is not necessary to assume that the absolute level of variability of the current assay is identical to that of the data used to determine the weighting, but only that the ratios of variances among concentrations are consistent with the historical data or the data used to determine the variance function.

Appropriate training and experience in statistical methods are essential in determining an appropriate variance-modeling strategy. Sources of variability may be misidentified if the wrong variance model is used. For example, data may have constant variation throughout a four-parameter logistic concentration–response curve but can also have appreciable variation in the EC_{50} parameter from block to block within the assay, or from assay to assay. If the between-block or between-assay variability is not recognized, this assay can appear to have large variation in the response for concentrations near the long-term average value of the EC_{50}. A weighted model with low weights for concentrations near the EC_{50} would misrepresent a major feature of such an assay system.

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4.4 Normality

Many statistical methods for the analysis of quantitative responses assume normality of the residuals. If the normality assumption is not met, the estimate of relative potency and its standard error may be reasonable, but suitability tests and a confidence interval for the relative potency estimate may be invalid. Most methods used in this chapter are reasonably robust to departures from normality, so the goal is to detect substantial nonnormality. During assay development, in order to discover substantial departure from normality, graphical tools such as a normal probability plot or a histogram (or something similar like stem-and-leaf or box plots) of the residuals from the model fit may be used. The histogram should appear unimodal and symmetric. The normal probability plot should approximate a straight line; a normal probability plot that is not straight (e.g., curved at one end, both ends, or in the middle) indicates the presence of nonnormality. A pattern about a straight line is an indication of nonnormality. Nonnormal behavior may be due to measurements that are log normal and show greater variability at higher levels of response. This may be seen as a concave pattern in the residuals in a normal plot.

Statistical tests of normality may not be useful. As per the previous discussion of statistical testing of constancy of variance, change of the value of a normality test statistic, rather than reliance on a p value, is useful for judging whether normality is improved or worsened. As for variance assessment, evaluate normality on as large a body of assay data as possible during development, re-assess during validation, and monitor periodically during ongoing use of the assay. Important departures from normality can often be mitigated with a suitable transformation. Failure to assess and mitigate important departure from normality carries the risks of disabling appropriate outlier detection and losing capacity to obtain reliable estimates of variation.

4.5 Linearity of Concentration–Response Data

Some bioassay analyses assume that the shape of the concentration–response curve is a straight line or approximates a straight line over a limited range of concentrations. In those cases, a linear-response model may be assessed to determine if it is justified for the data in hand. Difference testing methods for assessing linearity face the same problems as do difference testing methods applied to parallelism—more data and better precision make it more likely to detect nonlinearity. Because instances in which lack of linearity does not affect the potency estimate are rare, analysts should routinely assess departure from linearity if they wish to use a linear-response model to estimate potency.

If an examination of a data plot clearly reveals departure from linearity, this is sufficient to support a conclusion that linearity is not present. High data variability, however, may mask departure from linearity. Thus a general approach for linearity can conform to that for similarity, developed more elaborately in section 4.7 *Suitability Testing, Implementing Equivalence Testing for Similarity (parallelism)*.

1. Specify a measure of departure from linearity which can either combine across samples or be sample specific. Possibilities include the nonlinearity sum of squares or quadratic coefficients.
2. Use one of the four approaches in *Step 2* of *Implementing Equivalence Testing for Similarity (parallelism)* to determine, during development, a range of acceptable values (acceptance interval) for the measure of nonlinearity.
3. Determine a 90% two-sided confidence interval on the measure on nonlinearity, following the Two One-Sided Test (TOST) procedure, and compare the result to the acceptance interval as determined in (2).

Often a subset of the concentrations measured in the assay will be selected in order to establish a linear concentration–response curve. The subset may be identified graphically. The concentrations at the extreme ends of the range should be examined carefully as these often have a large impact on the slope and calculations derived from the slope. If, in the final assay, the intent is to use only concentrations in the linear range, choose a range of concentrations that will yield parallel straight lines for the relative potencies expected during routine use of the assay; otherwise, the assay will fail parallelism tests when the potency produces assay response values outside the linear range of response. When potency is outside the linear range, it may be appropriate to adjust the sample concentration based on this estimated potency and test again in order to obtain a valid potency result. The repeat assays together with the valid assays may generate a biased estimate of potency because of the selective process of repeating assays when the response is in the extremes of the concentration–response curve.

The problem is more complex in assays where there is even modest variation in the shape or location of the concentration–response curve from run to run or from block to block within an assay. In such assays it may be appropriate to choose subsets for each sample in each assay or even in each block within an assay. Note that a fixed-effects model will mask any need for different subsets in different blocks, but a mixed-effects model may reveal and accommodate different subsets in different blocks (see section 4.9 *Fixed and Random Effects in Models of Bioassay Response*).

Additional guidance about selection of data subset(s) for linear model estimation of relative potency includes the following: use at least three, and preferably four, adjacent concentrations; require that the slope of the linear segment is sufficiently steep; require that the lines fit to Standard and Test samples are straight; and require that the fit regression lines are parallel. One way to derive a steepness criterion is to compute a t-statistic on the slope difference from zero. If the slope is not significant the bioassay is likely to have poor performance; this may be observed as increased variation in the potency results. Another aspect that supports requiring adequate steepness of slope is the use of subset selection algorithms. Without a slope steepness criterion, a subset selection algorithm that seeks to identify subsets of three or more contiguous data points that are straight and parallel might select concentrations on an asymptote. Such subsets are obviously inappropriate to use for potency estimation. How steep or how significant the steepness of the slope should be depends on the assay. This criterion should be set during assay development and possibly refined during assay validation.

4.6 Common Bioassay Models

Most bioassays consist of a series of concentrations or dilutions of both a Test sample and a Standard material. A mathematical model is fit to the concentration–response data, and a relative potency may then be calculated from the parameters of the model. Choice of model may depend on whether quantitative or qualitative data are being analyzed.

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For quantitative data, models using parallel response profiles which support comparative evaluation for determining relative potency may provide statistical advantages. If such a model is used, concentrations or dilutions are usually scaled geometrically, e.g., usually in two-fold, log, or half-log increments. If a slope-ratio model is used, concentrations or dilutions can be equally spaced on concentration, rather than log concentration. Several functions may be used for fitting a parallel response model to quantitative data, including a linear function, a higher-order polynomial function, a four-parameter logistic (symmetric sigmoid) function, and a five-parameter logistic function for asymmetric sigmoids. Such functions require a sufficient number of concentrations or dilutions to fit the model. To assess lack of fit of any model it is necessary to have at least one, and preferably several, more concentrations (or dilutions) than the number of parameters that will be estimated in the model. Also, at least one, and better, two, concentrations are commonly used to support each asymptote.

A linear model is sometimes selected because of apparent efficiency and ease of processing. Because bioassay response profiles are usually nonlinear, the laboratory might perform an experiment with a wide range of concentrations in order to identify the approximately linear region of the concentration–response profile. For data that follow a four-parameter logistic model, these are the concentrations near the center of the response region, often from 20% to 80% response when the data are rescaled to the asymptotes. Caution is appropriate in using a linear model because for a variety of reasons the apparently linear region may shift. A stable linear region may be identified after sufficient experience with the assay and with the variety of samples that are expected to be tested in the assay. Data following the four-parameter logistic function may also be linearized by transformation. The lower region of the function is approximately linear when the data are log transformed (log–log fit).

Quantal data are typically fit using more complex mathematical models. A probit or logit model may be used to estimate a percentile of the response curve (usually the 50th percentile) or, more directly, the relative potency of the Test to the Standard. Spearman-Kärber analysis is a non-modeling method that may be employed for determining the 50th percentile of a quantal concentration–response curve.

4.7 Suitability Testing

System suitability and sample suitability assessment should be performed to ensure the quality of bioassay results. System suitability in bioassay, as in other analytical methods, consists of pre-specified criteria by which the validity of an assay (or, perhaps, a run containing several assays) is assessed. Analysts may assess system suitability by determining that some of the parameters of the Standard response are in their usual ranges and that some properties (e.g., residual variation) of the data are in their usual range. To achieve high assay acceptance rates, it is advisable to accept large fractions of these usual ranges (99% or more) and to assess system suitability using only a few uncorrelated Standard response parameters. The choice of system suitability parameters and their ranges may also be informed by empirical or simulation studies that measure the influence of changes in a parameter on potency estimation.

Sample suitability in bioassay is evaluated using pre-specified criteria for the validity of the potency estimate of an individual Test sample, and usually focuses on similarity assessment. System and sample suitability criteria should be established during bioassay development and before bioassay validation. Where there is limited experience with the bioassay, these criteria may be considered provisional.

SYSTEM SUITABILITY

System suitability parameters may be selected based on the design and the statistical model. Regardless of the design and model, however, system suitability parameters should be directly related to the quality of the bioassay. These parameters are generally based on standard and control samples. In parallel-line assays, for example, low values of the Standard slope typically yield estimates of potency with low precision. Rather than reject assays with low slope, analysts may find it more effective to use additional replicate assays until the assay system can be improved to consistently yield higher-precision estimates of potency. It may be particularly relevant to monitor the range of response levels and location of asymptotes associated with controls or Standard sample to establish appropriate levels of response. A drift or a trend in some of the criteria may indicate the degradation of a critical reagent or Standard material. Statistical process control (SPC) methods should be implemented to detect trends in system suitability parameters.

Two common measures of system suitability are assessment of the adequacy of the model (goodness of fit) and of precision. With replicates in a completely randomized design, a pure error term may be separated from the assessment of lack of fit. Care should be taken in deriving a criterion for lack of fit; the use of the wrong error term may result in an artificial assessment. The lack of fit sum of squares from the model fit to the Standard may, depending on the concentrations used and the way in which the data differ from the model, be a useful measure of model adequacy. A threshold may be established, based on *sensitivity analysis* (assessment of assay sensitivity to changes in the analyte) and/or historical data, beyond which the lack of fit value indicates that the data are not suitable. Note that the Test data are not used here; adequacy of the model for the Test is part of sample suitability.

For assessment of precision, two alternatives may be considered. One approach uses the mean squared error (residual variance) from the model fit to the Standard alone. Because this approach may have few degrees of freedom for the variance estimate, it may be more useful to use a pooled mean squared error from separate model fits to Standard and Test. Once the measure is selected, use historical data and sensitivity analysis to determine a threshold for acceptance.

SAMPLE SUITABILITY

Sample suitability in bioassay generally consists of the assessment of similarity, which can only be done within the assay range. Relative potency may be reported only from samples that both show similarity to Standard, exhibit requisite quality of model fit, and have been diluted to yield an EC_{50} (and potency) within the range of the assay system.

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SIMILARITY

In the context of similarity assessment, classical hypothesis (*difference*) testing evaluates a null hypothesis that a measure (a nonsimilarity parameter measuring the difference between Standard and Test concentration–response curves) is zero, with an implicit alternative hypothesis that the measure is non-zero or the statistical assumptions are not satisfied. The usual ("difference test") criterion that the p-value must be larger than a certain critical value in order to declare the sample similar to reference controls the probability that samples are falsely declared nonsimilar; this is the producer's risk of failing good samples. The consumer's risk (the risk that nonsimilar samples are declared similar) is controlled via the precision in the nonsimilarity measure and amount of replication in the assay; typically these are poorly assessed, leaving consumer risk uncontrolled.

In contrast to difference testing, equivalence testing for similarity (assessing whether a 90% confidence interval for a measure of nonsimilarity is contained within specified equivalence bounds) allows only a 5% probability that samples with nonsimilarity measures outside the equivalence boundaries will be declared similar (controlling the consumer's risk). With equivalence testing it is practical to examine and manage the producer's risk by ensuring that there is enough replication in the assay to have good precision in estimating the nonsimilarity measure(s).

For the comparison of slopes, difference tests have traditionally been used to establish parallelism between a Test sample and the Standard sample. Using this approach the laboratory cannot conclude that the slopes are equal. The data may be too variable, or the assay design may be too weak to establish a difference. The laboratory can, however, conclude that the slopes are sufficiently similar using the equivalence testing approach.

Equivalence testing has practical advantages compared to difference testing, including that increased replication yielding improved assay precision will increase the chances that samples will pass the similarity criteria; that decreased assay replication or precision will decrease the chances that samples will pass the similarity criteria; and that sound approaches to combining data from multiple assays of the same sample to better understand whether a sample is truly similar to Standard or not are obtained.

Because of the advantages associated with the use of equivalence testing in the assessment of similarity, analysts may transition existing assays to equivalence testing or may implement equivalence testing methods when changes are made to existing assays. In this effort, it is informative to examine the risk that the assay will fail good samples. This risk depends on the precision of the assay system, the replication strategy in the assay system, and the critical values of the similarity parameters (this constitutes a *process capability analysis*). One approach to transitioning an established assay from difference testing to equivalence testing (for similarity) is to use the process capability of the assay to set critical values for similarity parameters. This approach is reasonable for an established assay because the risks (of falsely declaring samples similar and falsely declaring samples nonsimilar) are implicitly acceptable, given the assay's history of successful use.

Similarity measures may be based on the parameters of the concentration–response curve and may include the slope for a straight parallel-line assay; intercept for a slope-ratio assay; the slope and asymptotes for a four-parameter logistic parallel-line assay; or the slope, asymptotes, and nonsymmetry parameter in a five-parameter sigmoid model. In some cases, these similarity measures have interpretable, practical meaning in the assay; certain changes in curve shape, for example, may be associated with specific changes (e.g., the presence of a specific active contaminant) in the product. When possible, discussion of these changes and their likely effects is a valuable part of setting appropriate equivalence boundaries.

IMPLEMENTING EQUIVALENCE TESTING FOR SIMILARITY (PARALLELISM)

As previously stated, many statistical procedures for assessing similarity are based on a null hypothesis stating that similarity is present and the alternative hypothesis of there being a state of nonsimilarity. Failure to find that similarity is statistically improbable is then taken as a conclusion of similarity. In fact, however, this failure to establish a probabilistic basis for nonsimilarity does not prove similarity. Equivalence testing provides a method for the analyst to proceed to a conclusion (if warranted by the data) of *sufficiently similar* while controlling the risk of doing so inappropriately. The following provides a sequence for this process of implementing equivalence testing.

Step 1: Choose a measure of nonsimilarity.

For the parallel-line case, this could be the difference or ratio of slopes. (The ratio of slopes can be less sensitive to the value of the slope. Framing the slope difference as a proportional change from Standard rather than in absolute slope units has an advantage because it is invariant to the units on the concentration and response axes.) For a slope-ratio assay, the measure of nonsimilarity can be the difference in y-intercepts between Test and Standard samples. Again, it can be advantageous to frame this difference as a proportion of the (possibly transformed) response range of Standard to make the measure invariant to the units of the response.

The determination of similarity could be based on the individual parameters, one at a time; for the four-parameter logistic model, similarity between Standard and Test samples can be assessed discretely for the upper asymptote, the slope, and the lower asymptote. If sigmoid curves with additional parameters are used to fit bioassay data, it is also important to consider addressing similarity between Standard and Test preparations of the additional curve parameters (e.g., asymmetry parameter of the five-parameter model). Alternatively, evaluation of similarity can be based on a single composite measure of nonparallelism, such as the *parallelism sum of squares*. This is found as the difference in residual sum of squared errors (RSSE) between the value obtained from fitting the Standard and Test curves separately and the value obtained from imposing parallelism:

$$\text{Parallelism sum of squares} = RSSE_p - RSSE_s - RSSE_t$$

where the subscripts P, S, and T denote Parallel model, Standard model, and Test model, respectively. With any composite measure, the analyst must consider the implicit relative weighting of the importance of the three (or more) curve regions and whether the weighting is appropriate for the problem at hand. For the parallelism sum of squares, for example, with nonlinear models, the weighting given to the comparison of the asymptotes depends on the amount of data in the current assay on and near the asymptotes.

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Step 2: Specify a range of acceptable values, typically termed an equivalence interval or "indifference zone," for the measure of nonsimilarity.

The challenge in implementing equivalence testing is in setting appropriate equivalence bounds for the nonsimilarity measures. Ideally, information is available to link variation in similarity measures to meaningful differences in biological function (as measured by the bioassay). Information may be available from evaluation of orthogonal assays. The following four approaches can be used to determine this interval. If pharmacopeial limits have been specified for a defined measure of nonsimilarity, then the assay should satisfy those requirements.

a. The first approach is to compile historical data that compare the Standard to itself and using these data to determine the equivalence interval as a tolerance interval for the measure of nonparallelism. The advantage of using historical data is that they give the laboratory control of the false failure rate (the rate of failing a sample that is in fact acceptable). The disadvantage is that there is no control of the false pass rate (the rate of passing a sample that may have an unacceptable difference in performance relative to the Standard). The equivalence interval specification developed in this way is based solely on assay capability. Laboratories that use this approach should take caution that an imprecise assay in need of improvement may yield such a wide equivalence interval that no useful discrimination of nonsimilarity is possible.

b. Approach (a) is simple to implement in routine use and can be used with assay designs that do not provide reliable estimates of within-assay variation and hence confidence intervals. However, there is a risk that assays with larger than usual amounts of within-assay variation can pass inappropriately. The preferable alternative to (a) is therefore to determine a tolerance interval for the confidence interval for the measure of nonparallelism. The following is particularly appropriate to transition an existing assay with a substantial body of historical data on both Standard and Test samples from a difference testing approach to an equivalence approach:

i. For each value of the measure of nonparallelism from the historical data, determine a 95% confidence interval, (m,n).

ii. For each confidence interval, determine its maximum departure from perfect parallelism. This is max(|m|, |n|) for differences, max(1/m,n) for ratios, and simply n for quantities that must be positive, such as a sum of squares.

iii. Determine a tolerance interval for the maximum departures obtained in (ii). This will be a one-sided tolerance interval for these necessarily positive quantities. A nonparametric tolerance interval approach is preferred.

iv. "Sufficiently parallel" is concluded for new data if the confidence interval for the measure of nonparallelism falls completely within the interval determined in (iii).

Approaches (a) and (b), through their reliance on assay capability, control only the false fail rate, and neglect the false pass rate. Incorporating information from sources other than the evaluation of assay capability provides control of the false pass rate. Approaches (c) and (d) are means to this end.

c. The third approach starts with historical data comparing the Standard to itself and adds data comparing the Standard to known failures, e.g., to degraded samples. Compare values of the measure of nonsimilarity for data for which a conclusion of similarity is appropriate (Standard against itself) and data for which a conclusion of similarity is not appropriate, e.g., degraded samples. Based on this comparison, determine a value of the measure of nonsimilarity that discriminates between the two cases. If this approach is employed, a range of samples for which a conclusion of similarity is not appropriate should be utilized, including samples with the minimal important nonsimilarity. For nonlinear models, this comparison also can be used to determine which parameters should be assessed; some may not be sensitive to the failures that can occur with the specific assay or collection of nonsimilar samples.

d. The fourth approach is based on combining a sensitivity analysis of the assay curve to nonsimilarity parameters with what is known about the product and the assay. It is particularly helpful if information is available that links a shift in one or more nonsimilarity measures to properties of the product. These measures may be direct (e.g., conformational changes in a protein) or indirect (e.g., changes in efficacy or safety in an animal model). A complementary approach is provided by a limited sensitivity analysis that combines analyst and biologist judgment regarding the magnitude of shifts in a nonsimilarity parameter that are meaningful, with simulation and/or laboratory experiments, to demonstrate thresholds for similarity parameters that provide protection against important nonsimilarity. Additionally, risk analysis may be informed by the therapeutic index of the drug.

Step 3. Examine whether the value of the nonsimilarity measure is found within the equivalence interval of acceptable values.

For approaches (a) and (b), compare the obtained value of the measure of nonparallelism (a) or its confidence interval (b) to the interval obtained at the beginning of Step 2. The value must be within the limits if one uses (a), or the confidence interval must be completely within the limits if one uses (b).

An alternative to the approach described above [for (a)] is to use an average (historical) value for the variance of the ratio or difference in a similarity parameter—obtained from some number of individual assays—to compute an acceptance interval for a point estimate of the similarity parameter. This approach is simpler to implement in routine use and can be used with assay designs that are unable to provide reliable estimates of within-assay variation. However, there is a price. The equivalence testing approach that relies on assay-specific (within-assay) measure(s) of variation (i.e., the confidence intervals) is conservative in the sense that it will fail to pass similarity for samples from assays that have larger than usual amounts of within-assay variation. Using an acceptance region for a similarity parameter—rather than an acceptance region for confidence intervals for the similarity parameter—loses this conservative property and hence is not preferred where alternatives exist.

For approach (c), an approach that essentially treats the parallelism as a discrimination problem may be used. The choice of the cut point in (c) should take into account the rates of false positive and false negative decisions (and the acceptable risks to the laboratory) and should reflect the between-assay variability in precision. Thus it is reasonable to compare the point estimate of the measure of nonparallelism to the cut point and to not use confidence intervals. This approach is simpler to implement in routine use and can be used with assay designs that cannot provide reliable estimates of within-assay variation.

For approach (d), demonstrate that the measure of nonsimilarity is significantly greater than the lower endpoint of the acceptance interval and significantly less than the upper endpoint. (If the acceptance interval is one-sided, then apply only the single applicable test.) This is use of the TOST approach. For most situations, TOST can be most simply implemented by calculating a 90% two-sided confidence interval, which corresponds to a 5% equivalence test. If this confidence interval lies

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entirely within the equivalence interval specified at the beginning of Step 2, then similarity is sufficiently demonstrated. For parallel-line models, one can use either (1) a confidence interval based on the value of the difference of the slopes ±k times the standard error of that value, or (2) Fieller's Theorem for the ratio of slopes may be used. For slope ratio models use the confidence interval for the difference of intercepts. For nonlinear models, there is evidence that these simple confidence interval methods do not attain the stated level of confidence, and methods based on likelihood profile or resampling are more appropriate.

RANGE

The range for a relative potency bioassay is the interval between the upper and lower relative potencies for which the bioassay is shown to have suitable levels of precision, relative accuracy, linearity of log potency, and success rates for system and sample suitability. It is straightforward to determine whether or not a sample that is similar to a Standard has a relative potency within the (validated) range of the assay system. For samples that are not similar according to established criteria, it is more challenging to determine whether a relative potency estimate for the sample might be obtained. In a nonlinear parallel-line assay a sample that does not have data on one asymptote might be assumed to be out of the potency range of the assay. In a parallel straight-line assay a sample that does not have three or more points on the steep portion of the response curve may be out of the potency range of the assay. For samples that have not been shown to be similar to reference it is not appropriate to report potency or to construct a ratio of EC_{50}s from unrestricted fits. As such samples may be out of the assay range, it may be useful to shift the dilution of the test sample for a subsequent assay on the basis of an estimate of relative activity. This estimated relative activity may be obtained via the ratio of the concentrations of Standard and Test that yields responses that match the reference response at the reference EC_{50}.

4.8 Outliers

Bioassay data should be screened for outliers before relative potency analysis. Outliers may be simple random events or a signal of a systematic problem in the bioassay. Systematic error that generates outliers may be due to a dilution error at one or more concentrations of a Test sample or the Standard or due to a mechanical error (e.g., system malfunction). Several approaches for outlier detection can be considered. Visual inspection is frequently utilized but should be augmented with a more objective approach to avoid potential bias.

An outlier is a datum that appears not to belong among the other data present. An outlier may have a distinct, identifiable cause, such as a mistake in the bench work, equipment malfunction, or a data recording error, or it could just be an unusual value relative to the variability typically seen and may appear without an identifiable cause. The essential question pertaining to an outlier becomes: Is the apparent outlier sampled from the same population as the other, less discordant, data, or is it from another population? If it comes from the same population and the datum is, therefore, an unusual (yet still legitimate) value obtained by chance, then the datum should stand. If it comes from another population and the datum's excursive value is due to human error or instrument malfunction, then the datum should be omitted from calculations. In practice, the answer to this essential question is often unknown, and investigations into causes are often inconclusive. Outlier management relies on procedures and practices to yield the best answer possible to that essential question and to guide response accordingly.

General chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses outlier labeling, identification, and rejection; statistical methods are included. General chapter ⟨1010⟩ also lists additional sources of information that can provide a comprehensive review of the relevant statistical methodology. General chapter ⟨1010⟩ makes no explicit remarks regarding outlier analysis in linear or nonlinear regression. Outlier analysis techniques appropriate for data obtained from regression of response on concentration can be used. Some remarks about outliers are provided here in the context of bioassays to emphasize or complement the information in ⟨1010⟩.

Of the procedures employed for analysis of drug compounds and biological drugs, the bioassay may be expected to be the most prone to outlying data. The management of outliers is appropriate with bioassay data on at least two levels: where an individual datum or a group of data (e.g., data at a concentration) can be checked against expected responses for the sample and concentration; and, separately, when estimates of relative potency from an assay can be checked for consistency with other independent estimates of the potency of the same material.

Three important aspects of outlier management are prevention, labeling, and identification.

Outlier prevention is preferred for obvious reasons, and is facilitated by procedures that are less subject to error and by checks that are sensitive to the sorts of errors that, given the experience gained in assay development, may be expected to occur. In effect, the error never becomes an outlier because it is prevented from occurring.

Good practice calls for the examination of data for outliers and labeling ("flagging") of the apparently outlying observation(s) for investigation. If investigation finds a cause, then the outlying datum may be excluded from analysis. Because of the ordinary occurrence of substantial variability in bioassay response, a laboratory's investigation into the outlying observation is likely to yield no determinable cause. However, the lack of evidence regarding an outlier's cause is not a clear indication that statistical outlier testing is warranted. Knowledge of the typical range of assay response variability should be the justification for the use of statistical outlier tests.

Outlier identification is the use of rules to confirm that the values are inconsistent with the known or assumed statistical model. For outliers with no determined cause, it is tempting to use statistical outlier identification procedures to discard unusual values. Discarding data solely because of statistical considerations should be a rare event. Falsely discarding data leads to overly optimistic estimates of variability and can bias potency estimates. The laboratory should monitor the failure rate for its outlier procedure and should take action when this is significantly higher than expected.

Statistical procedures for outlier identification depend on assumptions about the distribution of the data without outliers. Identification of data as outliers may mean only that the assumption about distribution is not correct. If dropping outliers because of statistical considerations is common, particularly if outliers tend to occur more often at high values or at high responses, then this may be an indication that the data require some adjustment, such as log transformation, as part of the assay procedure. Two approaches to statistical assessment of outlying data are replication-based and model-based.

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REPLICATION-BASED APPROACHES

When replicates are performed at concentrations of a Test sample and the Standard, an "extra variability" (EV) criterion may be employed to detect outliers. Historical data can be analyzed to determine the range in variability commonly observed among replicates, and this distribution of ranges can be used to establish an extreme in the range that might signal an outlier. Metrics that can be utilized are the simple range (maximum replicate minus minimum replicate), the standard deviation, or the CV or RSD among replicates. However, if the bioassay exhibits heterogeneity of variability, assumptions about uniform scatter of data are unsupported. Analysts can use a variable criterion across levels in the bioassay, or they can perform a transformation of the data to a scale that yields homogeneity of variability. Transformation can be performed with a POM approach as discussed previously. Where heterogeneity exists nonnormality is likely present, and the range rather than standard deviation or RSD should be used.

The actions taken upon detection of a potential outlier depend in part on the number of replicates. If EV is detected within a pair (n = 2) at a concentration of a Test sample or the Standard, it will not always be clear which of the replicates is aberrant, and the laboratory should eliminate the concentration from further processing. If more than two replicates are performed at each dilution the laboratory may choose to adopt a strategy that identifies which of the extremes may be the outlier. Alternatively, the laboratory may choose to eliminate the dilution from further processing.

MODEL-BASED APPROACHES

Model-based approaches may be used to detect outliers within bioassay data. These approaches use the residuals from the fit of an appropriate model. In general, if using model-based methods to identify potential outliers, the models used may make fewer assumptions about the data than the models used to assess suitability and estimate potency. For example, a non-parametric regression (smoothing) model may be useful.

Lastly, an alternative to discarding outlying data is to use robust methods that are less sensitive to influence by outlying observations. Use of the median rather than the mean to describe the data's center exemplifies a robust perspective. Also, regression using the method of least squares, which underlies many of the methods in this chapter, is not robust in the presence of outliers. The use of methods such as robust regression may be appropriate but is not covered in the USP bioassay chapters.

4.9 Fixed and Random Effects in Models of Bioassay Response

The choice of treating factors as fixed or random is important for the bioassay design, the development experiments, the statistical analysis of data, and the bioassay validation. Fixed effects are factors for which all levels, or all levels of interest, are discretely present, like sample, concentration, temperature and duration of thaw, and incubation time. Data for a response at some level, or combination of levels, of a fixed factor, can predict future responses. Fixed effects are expected to cause a consistent shift in responses. Analysts study fixed effects by controlling them in the design and examining changes in means across levels of the factor.

Random effects are factors of which the levels in a particular run of an assay are considered representative of levels that could be present. That is, there is no expectation that any specific value of the random factor will influence response. Rather, that value may vary subject to some expected distribution of values and thus may be a source of variability. For example, there is no desire to predict assay response for a specific day, but there is interest in predicting the variation in response associated with the factor "day". Examples of random effects include reagent lot, operator, or day if there is no interest in *specific* reagent lots, operators, or day as sources of variability. Analysts may study random effects by measuring the variance components corresponding to each random effect. Variance components can be estimated well only if there are an appreciable number of levels of each random effect. If there are, for example, only two or three reagent lots or analysts present, the variation associated with these factors will be poorly estimated.

Making a correct choice regarding treating a factor as fixed or random is important to the design of the assay and to proper reporting of its precision. Treating all factors as fixed, for example, leads to an understatement of assay variability because it ignores all sources of variability other than replication. The goal is to identify specific sources of variability that can be controlled, to properly include those factors in the design, and then to include other factors as random.

If the factor may switch from random to fixed effect or vice versa, the factor should normally be modeled as a random effect. For example, reagent lots cannot be controlled, so different lots are typically considered to cause variability, and reagent lot would be considered a random effect. However, if a large shift in response values has been traced to a particular lot, a comparison among a set of lots could be performed using reagent lot as a fixed effect. Similarly, within-assay location (e.g., block, plate, plate row, plate column, or well) or sequence may be considered a source of random variation or a source of a consistent (fixed) effect.

Assay designs that consist of multiple factors are efficient, but require corresponding statistical techniques that incorporate the factors as fixed or random effects in the analysis. If all factors are fixed, the statistical model is termed a fixed-effects model. If all are random, it is termed a random-effects model. If some factors are fixed and some random, the model is a mixed-effects model. Note that the concepts of fixed and random effects apply to models for quantitative, qualitative and integer responses. For assay designs that include multiple experimental units (e.g., samples assigned to sets of tubes and concentrations assigned to pre-plate tubes) a mixed-effects model in which the experimental units are treated as random effects is particularly effective. Additional complexity is added by the presence of designs with crossed random effects (e.g., each operator used material from one or more reagent batches, but many reagent batches were used by multiple operators). This can cause methodological and computational challenges for model fitting, especially when the designs are unbalanced.

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5. STAGES IN THE BIOASSAY DEVELOPMENT PROCESS

Given the ubiquity of cell-based assays and the motivation to use one bioassay system to provide context for discussion, the development of a cell-based bioassay will be used to illustrate the stages in the bioassay development continuum.

5.1 Design: Assay Layout, Blocking, and Randomization

Most cell-based assays are performed using a cell culture plate (6-, 12-, 96-, or 384-well micro titer plate). Ideally, a plate is able to provide a uniform substrate for experimental treatments in all wells, including after wash steps and incubations. However, regardless of assay conditions intended to minimize the potential for bias (e.g., good analyst technique, careful calibration of pipets, controlled incubation time, and temperature), systematic gradients on the plate, independent of experimental treatments, may be observed. These gradients may occur across rows, across columns, or from the edge to the center of the plate and are often called *plate effects.* Even moderate or inconsistent plate effects should be addressed during assay development, by means of plate layout strategies, blocking, randomization, and replication.

Plate effects can be evaluated in a *uniformity trial* in which a single experimental treatment, such as an assay concentration chosen from the middle section of the concentration–response curve, is used in all wells of the plate. *Figure 1* provides an example of what may be observed; a trend of decreasing signal is evident from right to left. In this case, it was discovered that the plate washer was washing more briskly on the left side of the plate, and required adjustment to provide uniform washing intensity and eliminate the gradient. Another common plate effect is a differential cell-growth pattern in which the outer wells of the plate grow cells in such a way that the assay signal is attenuated. This is such a persistent problem that the choice is often made to not use the outer wells of the assay plate. Because location effects are so common, designs that place replicates (e.g., of sample by concentration combinations) in adjacent wells should be avoided.

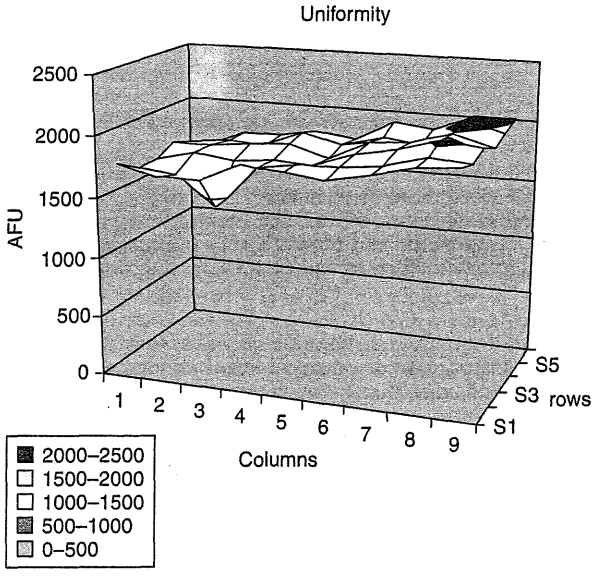

Figure 1. Plot of change in assay response across a plate.

Blocking is the grouping of related experimental units in experimental designs. Blocks may consist of individual 96-well plates, sections of 96-well plates, or 96-well plates grouped by analyst, day, or batch of cells. The goal is to isolate any systematic effects so that they do not obscure the effects of interest. A *complete block design* occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) are applied to experimental units for that factor within a single block. An *incomplete block design* occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

Randomization is a process of assignment of treatment to experimental units based on chance so that all such experimental units have an equal chance of receiving a given treatment. Although challenging in practice, randomization of experimental treatments has been advocated as the best approach to minimizing assay bias or, more accurately, to protecting the assay results from known and unknown sources of bias by converting bias into variance. While randomization of samples and concentrations to individual plate wells may not be practical, a plate layout can be designed to minimize plate effects by alternating sample positions across plates and the pattern of dilutions within and across plates. Where multiple plates are required in an assay, the plate layout design should, at a minimum, alternate sample positions across plates within an assay run to accommodate possible bias introduced by the analyst or equipment on a given day. It is prudent to use a balanced rotation of layouts on plates so that the collection of replicates (each of which uses a different layout) provides some protection against likely sources of bias.

Figure 2 illustrates a patterned assay design that lacks randomization and is susceptible to bias. Dilutions and replicates of the Test preparations (A and B) and the Standard (R) are placed together sequentially on the plate. Bias due to a plate or

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incubator effect can influence some or all of the concentrations of one of the samples. Note that in *Figures 2* through *5* all outer plate wells are left as blanks to protect against *edge effect.*

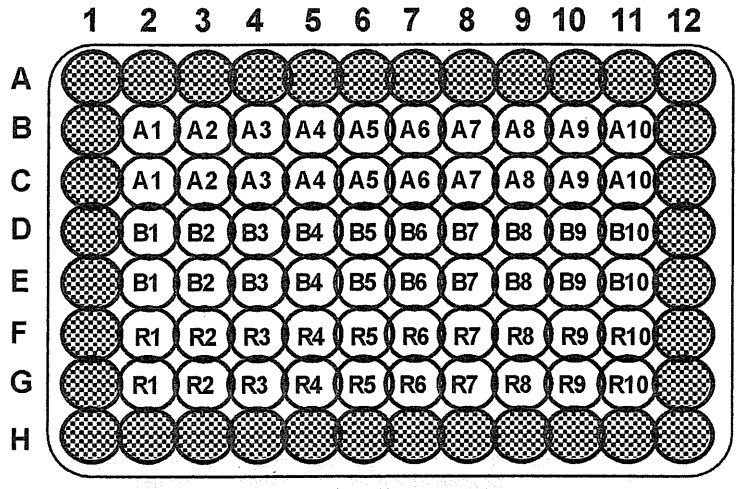

Figure 2. A highly patterned plate.

A layout that provides some protection from plate effects and can be performed manually is a *strip-plot design,* shown in *Figure 3*. Here samples are randomized to rows of a plate and dilution series are performed in different directions in different sections (blocks) on the plate to mitigate bias across columns of the plate. An added advantage of the strip-plot design is the ability to detect location effects by the interaction of sample and dilution direction (left-to-right or right-to-left).

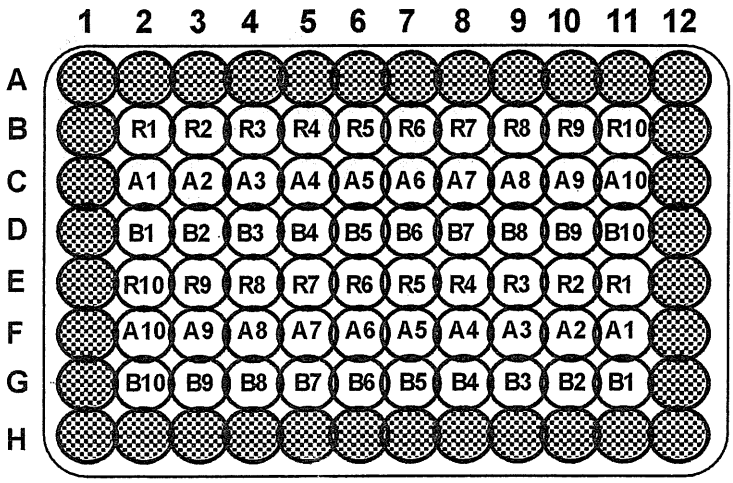

Figure 3. A strip-plot design.

Figure 4 illustrates an alternation of Test (Test sample 1 = "1"; Test sample 2 = "2") and Standard ("R") positions on multiple plates, within a single assay run; this protects against plate row effects. Combining the two methods illustrated if *Figures 3* and *4* can effectively help convert plate bias into assay variance. Assay variance may then be addressed, as necessary, by increased assay replication (increased number of plates in an assay).

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Plate Row	Plate 1	Plate 2	Plate 3
B	R	2	1
C	1	R	2
D	2	1	R
E	R	2	1
F	1	R	2
G	2	1	R

Figure 4. A multi-plate assay with varied Test and Reference positions.

A *split-plot design,* an alternative that assigns samples to plate rows randomly and randomizes dilutions (concentrations) within each row, is seen in *Figure 5*. Such a strategy may be difficult to implement even with the use of robotics.

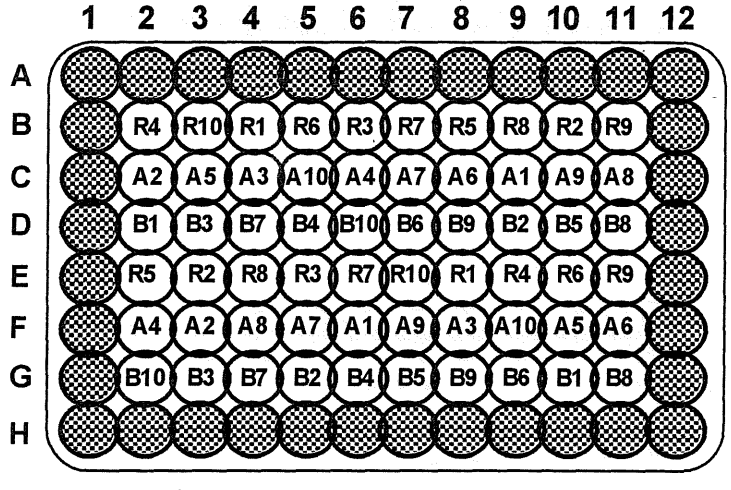

Figure 5. A split-plot design.

Dilution Strategy

Assay concentrations of a Test sample and the Standard can be obtained in different ways. Laboratories often perform serial dilutions, in which each dilution is prepared from the previous one, in succession. Alternatively, the laboratory may prepare wholly independent dilutions from the Test sample and Standard to obtain independent concentration series. These two strategies result in the same nominal concentrations, but they have different properties related to error. Serial dilutions are subject to propagation of error across the dilution series, and a dilution error made at an early dilution will result in correlated, non-independent observations. Correlations may also be introduced by use of multichannel pipets. Independent dilutions help mitigate the bias resulting from dilution errors.

It is noteworthy that when working to improve precision, the biggest reductions in variance come when replicating at the highest possible levels of nested random effects. This is particularly effective when these highest levels are sources of variability. To illustrate: replicating extensively within a day for an assay known to have great day-to-day variation is not effective in improving precision of reportable values.

5.2 Development

A goal of bioassay development is to achieve optimal bioassay relative accuracy and precision of the potency estimate. An endpoint of assay development is the completed development of the assay procedure, a protocol for the performance of the bioassay. The procedure should include enough detail so that a qualified laboratory with a trained analyst can perform the procedure in a routine manner. A strategic part of development is a look forward toward performance maintenance. Standard operating procedures for reagent and technician qualification, as well as for calibration of the working Standard, help complete the bioassay development package.

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ONE FACTOR AT A TIME VERSUS DESIGN OF EXPERIMENTS

Bioassay development proceeds through a series of experiments in which conditions and levels of assay factors are varied to identify those that support a reliable and robust bioassay qualified for routine use. Those experiments may be conducted one factor at a time (OFAT), studying each parameter separately to identify ideal conditions, or through the use of multi-factor design of experiments (DOE). DOE is an efficient and effective strategy for developing a bioassay and improving bioassay performance, thus helping to obtain a measurement system that meets its requirements. In comparison to OFAT, DOE generally requires fewer experiments and also provides insight into interactions of factors that affect bioassay performance. Assay development using DOE may proceed through a series of steps: process mapping and risk analysis; screening; response optimization; and confirmation.

PROCESS MAPPING AND RISK ANALYSIS

Bioassay optimization may begin with a systematic examination and risk assessment to identify those factors that may influence bioassay response. It is useful to visualize bioassay factors using a bioassay process map such as a cause-and-effect or fishbone diagram. Using the process map as a guide, the laboratory can examine assay factors that might affect assay performance, such as buffer pH, incubation temperature, and incubation time. Historical experience with one or several of the bioassay steps, along with sound scientific judgment, can identify key factors that require further evaluation. One tool that may be used to prioritize factors is a failure mode and effects analysis. Factors are typically scored by the combination of their potential to influence assay response and the likelihood that they will occur. The laboratory must be careful to recognize potential interactions between assay factors.

SCREENING

Once potential key factors have been identified from process mapping and risk analysis, the laboratory may conduct an initial screening experiment to probe for effects that may require control. Screening designs such as factorial and fractional factorial designs are commonly used for this purpose. Software is available to assist the practitioner in the selection of the design and in subsequent analysis. Analysts should take care, however, to understand their assumptions about design selection and analysis to ensure accurate identification of experimental factors.

RESPONSE OPTIMIZATION

A screening design will usually detect a few important factors from among those studied. Such factors can be further studied in a response-optimization design. Response-optimization designs such as central composite designs are performed to determine optimal settings for combinations of bioassay factors for achieving desired response. The information obtained from response optimization may be depicted as a response surface and can be used to establish ranges that yield acceptable assay performance and will be incorporated into the bioassay procedure.

In the parlance of Quality by Design (QbD), the "region" where the combined levels of input variables and process parameters have been demonstrated to provide acceptable assay performance is described as the *design space* for the bioassay. Establishing a true design space for a bioassay is challenging; some but not all factors and levels of random factors will be included in the development DOE, and there is no assurance that the design space is not sensitive to unstudied random factors. Similarly, there is little assurance that the assay (design space) is robust to random factors that are studied using small samples (or non-random samples of levels). Elements of DOE that may be considered include the use of blocks; deliberate confounding among interactions that are of lower interest, or known to be unimportant; robust design (response surface designs with random effects); and use of split-plot, strip-plot, or split-lot designs.

CONFIRMATION

The mathematical model depicting assay performance as a function of changes in key assay factors is an approximation; thus, it is customary to confirm performance at the ideal settings of the bioassay. Confirmation can take the form of a qualification trial in which the assay is performed, preferably multiple independent times using optimal values for factors. Alternatively, the laboratory may determine that the bioassay has been adequately developed and may move to validation. Qualification is a good practice, not a regulatory requirement. The decision to perform confirmatory qualifying runs or to proceed to validation depends upon the strength of the accumulated information obtained throughout development.

5.3 Data Analysis during Assay Development

Analysis of bioassay data during assay development enables analysts to make decisions regarding the statistical model that will be used for routine analysis, including transformation and/or weighting of data, and the development of system and sample suitability criteria. The analysis also provides information regarding which elements of design structure should be used during outlier detection and the fitting of a full model. This may also include a plan for choosing subsets of data, such as a linear portion, for analysis or, for nonlinear bioassays, a model reduction strategy for samples similar to Standard. Once these decisions are made and proven sound during validation, they don't need to be reassessed with each performance of the assay. A process approach to enabling these decisions follows.

Step 1: Choose an appropriate statistical model (also see section 4.6 Common Bioassay Models).
Given the complexity of bioassays and the motivation to use an approach proven reliable, fairly standardized analytical models are common in the field of bioassay analysis. Nonetheless, many considerations are involved in choosing the most appropriate statistical model. First, the model should be appropriate for the type of assay endpoint—continuous, count, or dichotomous. Second, the model should incorporate the structure of the assay design. For any design other than

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completely randomized, there will be terms in the model for the structural elements. These could be, for example, within-plate blocking, location of cage in the animal facility, day, etc. A third consideration, applicable to continuous endpoints, involves whether to use a regression model or a means model (an analysis of variance model that fits a separate mean at each dilution level of each sample tested), with appropriate error terms. A means model can be appropriate at this stage because it makes no assumptions about the shape of the concentration–response curve.

Step 2: Fit the chosen statistical model to the data without the assumption of parallelism, and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance.

Transform the data as necessary or, if needed, choose a weighting scheme (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation*). Use as large a body of assay data, from independent assays, as possible. The primary goal is to address any departure from normality and from constant variance of responses across the range of concentrations in the assay. Step 2 will likely alternate between imposing a transformation and assessing the distribution of the residuals.

Step 3: Screen for outliers, and remove as is appropriate.

This step normally follows the initial choice of a suitable transformation and/or weighting method. Ideally the model used for outlier detection contains the important elements of the assay design structure, allows nonsimilar curves, and makes fewer assumptions about the functional shape of the concentration–response curve than did the model used to assess similarity. See section 4.8 *Outliers* and general chapter ⟨1010⟩ for discussion of outlier detection and removal. In some cases, outliers may be so severe that a reasonable model cannot be fit, and thus residuals will not be available. In such cases, it is necessary to screen the raw data for outliers before attempting to fit the model.

During assay development, a strategy should be developed for the investigation and treatment of an outlier observation, including any limits on how many outliers are acceptable. Include these instructions in the assay SOP. Good practice includes recording the process of an investigation, outlier test(s) applied, and results therefrom. Note that outlier procedures must be considered apart from the investigation and treatment of an out-of-specification (OOS) result (reportable value). Decisions to remove an outlier from data analysis should not be made on the basis of how the reportable value will be affected (e.g., a potential OOS result). Removing data as outliers should be rare. If many values from a run are removed as outliers, that run should be considered suspect.

Step 4: Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.

Step 5: If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear (see section 4.5 Linearity of Concentration–Response Data).

Step 6: Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.

5.4 Bioassay Validation

The bioassay validation is a protocol-driven study that demonstrates that the procedure is fit for use. A stage-wise approach to validation may be considered, as in a "suitable for intended use" validation to support release of clinical trial material, and a final, comprehensive validation prior to BLA or MAA filing. Preliminary system and sample suitability controls should be established and clearly described in the assay procedure; these may be finalized based on additional experience gained in the validation exercise. Chapter ⟨1033⟩ provides validation comprehensive discussion of bioassay validation.

5.5 Bioassay Maintenance

The development and validation of a bioassay, though discrete operations, lead to ongoing activities. Assay improvements may be implemented as technologies change, as the laboratory becomes more skilled with the procedure, and as changes to bioassay methodology require re-evaluation of bioassay performance. Some of these changes may be responses to unexpected performance during routine processing. Corrective action should be monitored using routine control procedures. Substantial changes may require a study verifying that the bioassay remains fit for use. An equivalence testing approach can be used to show that the change has resulted in acceptable performance. A statistically-oriented study can be performed to demonstrate that the change does not compromise the previously acceptable performance characteristics of the assay.

ASSAY TRANSFER

Assay transfer assumes both a known intended use of the bioassay in the recipient lab and the associated required capability for the assay system. These implicitly, though perhaps not precisely, demarcate the limits on the amount of bias and loss of precision allowed between labs. Using two laboratories interchangeably to support one product will require considering the variation between labs in addition to intermediate precision for sample size requirements to determine process capability. For a discussion and example pertaining to the interrelationship of bias, process capability, and validation, see *A Bioassay Validation Example* in ⟨1033⟩.

IMPROVING OR UPDATING A BIOASSAY SYSTEM

A new version of a bioassay may improve the quality of bias, precision, range, robustness, specificity, lower the operating costs or offer other compelling advantages. When improving or updating a bioassay system a bridging study may be used to compare the performance of the new to the established assay. A wide variety of samples (e.g., lot release, stability, stressed, critical isoforms) can be used for demonstrating equivalence of estimated potencies. Even though the assay systems may be quite different (e.g., an animal bioassay versus a cell-based bioassay), if the assays use the same Standard and mechanism of action, comparable potencies may reasonably be expected. If the new assay uses a different Standard, the minimum requirement for an acceptable comparison is a unit slope of the log linear relationship between the estimated potencies. An important

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implication of this recommendation is that poor precision or biased assays used early can have lasting impact on the replication requirements, even if the assay is later replaced by an improved assay.

⟨1033⟩ BIOLOGICAL ASSAY VALIDATION

1. INTRODUCTION

Biological assays (also called bioassays) are an integral part of the quality assessment required for the manufacturing and marketing of many biological and some non-biological drug products. Bioassays commonly used for drug potency estimation can be distinguished from chemical tests by their reliance on a biological substrate (e.g., animals, living cells, or functional complexes of target receptors). Because of multiple operational and biological factors arising from this reliance on biology, they typically exhibit a greater variability than do chemically-based tests.

Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes of a biological drug product. As described in the ICH Guideline entitled Specifications: Test Procedures And Acceptance Criteria For Biotechnological/Biological Products (Q6B), section 2.1.2, bioassay techniques may measure an organism's biological response to the product; a biochemical or physiological response at the cellular level; enzymatic reaction rates or biological responses induced by immunological interactions; or ligand- and receptor-binding. As new biological drug products and new technologies emerge, the scope of bioassay approaches is likely to expand. Therefore, general chapter *Biological Assay Validation* ⟨1033⟩ emphasizes validation approaches that provide flexibility to adopt new bioassay methods, new biological drug products, or both in conjunction for the assessment of drug potency.

Good manufacturing practice requires that test methods used for assessing compliance of pharmaceutical products with quality requirements should meet appropriate standards for accuracy and reliability. Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. *USP* general chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1) describe the assay performance characteristics (parameters) that should be evaluated for procedures supporting small-molecule pharmaceuticals. Although evaluation of these validation parameters is straightforward for many types of analytical procedures for well-characterized, chemically-based drug products, their interpretation and applicability for some types of bioassays has not been clearly delineated. This chapter addresses bioassay validation from the point of view of the measurement of activity rather than mass or other physicochemical measurements, with the purpose of aligning bioassay performance characteristics with uses of bioassays in practice.

Assessment of bioassay performance is a continuous process, but bioassay validation should be performed when development has been completed. Bioassay validation is guided by a validation protocol describing the goals and design of the validation study. General chapter ⟨1033⟩ provides validation goals pertaining to *relative potency* bioassays. Relative potency bioassays are based on a comparison of bioassay responses for a Test sample to those of a designated Standard that provides a quantitative measure of the Test bioactivity relative to that of the Standard.

Validation parameters discussed include *relative accuracy, specificity, intermediate precision,* and *range.* Laboratories may use *dilutional linearity* to verify the *relative accuracy* and *range* of the method. Although robustness is not a requirement for validation, general chapter ⟨1033⟩ recommends that a bioassay's robustness be assessed prior to validation. In addition, ⟨1033⟩ describes approaches for validation design (sample selection and replication strategy), validation acceptance criteria, data analysis and interpretation, and finally bioassay performance monitoring through quality control. Documentation of bioassay validation results is also discussed, with reference to pre-validation experiments performed to optimize bioassay performance. In the remainder of general chapter ⟨1033⟩ the term "bioassay" should be interpreted as meaning "relative potency bioassay".

2. FUNDAMENTALS OF BIOASSAY VALIDATION

The goal of bioassay validation is to confirm that the operating characteristics of the procedure are such that the procedure is suitable for its intended use. The issues involved in developing a bioassay are described in greater detail in general chapter ⟨1032⟩ and are assumed resolved by the time the bioassay is in validation. Included in those decisions will be identification of what constitutes an assay and a run for the bioassay. Multiple dilutions (concentrations) of the Standard and one or more Test samples constitute a *replicate set* (also known as a minimal set), which contain a test substrate (e.g., group of animals or vessel of cells) at each dilution for each sample [Test(s) and Standard]. A *run* is defined as work performed during a period when the accuracy (trueness) and precision in the assay system can reasonably be expected to be stable. In practice, a run frequently consists of the work performed by a single analyst in one lab, with one set of equipment, in a short period of time (typically a day). An assay is the body of data used to assess similarity and estimate potency relative to a Standard for each Test sample in the assay. A run may contain multiple assays, a single assay, or part of an assay. Multiple assays may be combined to yield a reportable value for a sample. The reportable value is the value that is compared to a product specification.

In assays that involve groups at each dilution (e.g., 6 samples, each at 10 dilutions, in the non-edge wells of each of several 96-well cell culture plates) the groups (plates) constitute statistical *blocks* that should be elements in the assay and validation analyses (blocks are discussed in ⟨1032⟩). Within-block replicates for Test samples are rarely cost-effective. Blocks will not be further discussed in this chapter; more detailed discussion is found in ⟨1032⟩.

The amount of activity (potency) of the Standard is initially assigned a value of 1.0 or 100%, and the potency of the Test sample is calculated by comparing the concentration–response curves for the Test and Standard pair. This results in a unitless measure, which is the relative potency of the Test sample in reference to the potency of the Standard. In some cases the Standard is assigned a value according to another property such as protein concentration. In that case the potency of the Test sample is the relative potency times the assigned value of the Standard. An assumption of parallel-line or parallel-curve (e.g., four-parameter logistic) bioassays is that the dose–response curves that are generated using a Standard and a Test sample have

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similar (parallel) curve shape distinguished only by a horizontal shift in the log dose. For slope-ratio bioassays, curves generated for Standard and Test samples should be linear, pass through a common intercept, and differ only by their slopes. Information about how to assess parallelism is provided in general chapters ⟨1032⟩ and ⟨1034⟩.

In order to establish the *relative accuracy* and *range* of the bioassay, validation Test samples may be constructed using a dilution series of the Standard to assess dilutional linearity (linearity of the relationship between known and measured relative potency). In addition, the validation study should yield a representative estimate of the variability of the relative potency determination. Although robustness studies are usually performed during bioassay development, key factors in these studies such as incubation time and temperature and, for cell-based bioassays, cell passage number and cell number may be included in the validation, particularly if they interact with another factor that is introduced during the validation (e.g., a temperature sensitive reagent that varies in its sensitivity from lot-to-lot). Because of potential influences on the bioassay from inter-run factors such as multiple analysts, instruments, or reagent sources, the design of the bioassay validation should include consideration of these factors. The variability of potency from these combined elements defines the *intermediate precision* (IP) of the bioassay. An appropriate study of the variability of the potency values obtained, including the impact of intra-assay and inter-run factors, can help the laboratory confirm an adequate testing strategy and forecast the inherent variability of the *reportable value* (which may be the average of multiple potency determinations). Variability estimates can also be utilized to establish the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. (See section 3.4 *Use of Validation Results for Bioassay Characterization*.)

Demonstrating specificity (also known as selectivity) requires evidence of lack of influence from matrix components such as manufacturing process components or degradation products so that measurements quantify the target molecule only. Other analytical methods may complement a bioassay in measuring or identifying other components in a sample.

2.1 Bioassay Validation Protocol

A bioassay validation protocol should include the number and types of samples that will be studied in the validation; the study design, including inter-run and intra-run factors; the replication strategy; the intended validation parameters and justified target acceptance criteria for each parameter; and a proposed data-analysis plan. Note that in regard to satisfying acceptance criteria, failure to find a statistically significant effect is not an appropriate basis for defining acceptable performance in a bioassay; conformance to acceptance criteria may be better evaluated using an equivalence approach.

In addition, assay, run, and sample acceptance criteria such as system suitability and similarity should be specified before performing the validation. Depending on the extent of development of the bioassay, these may be proposed as tentative and can be updated with data from the validation. Assay, run, or sample failures may be reassessed according to criteria which have been defined in the validation protocol and, with sound justification, included in the overall validation assessment. Additional validation trials may be required in order to support changes to the method.

The bioassay validation protocol should include target acceptance criteria for the proposed validation parameters. Steps to be taken upon failure to meet a target acceptance criterion should be specified in the validation protocol, and may result in a limit on the range of potencies that can be measured in the bioassay or a modification to the replication strategy in the bioassay procedure.

2.2 Documentation of Bioassay Validation Results

Bioassay validation results should be documented in a bioassay validation report. The validation report should support the conclusion that the method is fit for use or should indicate corrective action (such as an increase in the replication strategy) that will be undertaken to generate sufficiently reliable results to achieve fitness for use. The report could include the raw data and intermediate results (e.g., variance component estimates should be provided in addition to overall intermediate precision) which would facilitate reproduction of the bioassay validation analysis by an independent reviewer. Estimates of validation parameters should be reported at each level and overall as appropriate. Deviations from the validation protocol should be documented with justification. The conclusions from the study should be clearly described with references to follow-up action as necessary. Follow-up action can include amendment of system or sample suitability criteria or modification of the bioassay replication strategy. Reference to prevalidation experiments may be included as part of the validation study report. Prevalidation experiments may include robustness experiments, where bioassay parameters have been identified and ranges have been established for significant parameters, and also may include qualification experiments, where the final procedure has been performed to confirm satisfactory performance in routine operation. Conclusions from prevalidation and qualification experiments performed during development contribute to the description of the operating characteristics of the bioassay procedure.

2.3 Bioassay Validation Design

The biological assay validation should include samples that are representative of materials that will be tested in the bioassay and should effectively establish the performance characteristics of the procedure. For *relative accuracy,* sample relative potency levels that bracket the range of potencies that may be tested in the bioassay should be used. Thus samples that span a wide range of potencies might be studied for a drug or biological with a wide specification range or for a product that is inherently unstable, but a narrower range can be used for a more durable product. A minimum of three potency levels is required, and five are recommended for a reliable assessment. If the validation criteria for relative accuracy and IP are satisfied, the potency levels chosen will constitute the *range* of the bioassay. A limited range will result from levels that fail to meet their target acceptance criteria. Samples may also be generated for the bioassay validation by stressing a sample to a level that might be observed in routine practice (i.e., stability investigations). Additionally, the influences of the sample matrix (excipients, process constituents, or combination components) can be studied strategically by intentionally varying these together with the target

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analyte, using a multifactorial approach. Often this will have been done during development, prior to generating release and stability data.

The bioassay validation design should consider all facets of the measurement process. Sources of bioassay measurement variability include sample preparation, intra-run factors, and inter-run factors. Representative estimation of bioassay variability necessitates consideration of these factors. Test sample and Standard preparation should be performed independently during each validation run.

The replication strategy used in the validation should reflect knowledge of the factors that might influence the measurement of potency. Intra-run variability may be affected by bioassay operating factors that are usually set during development (temperature, pH, incubation times, etc.); by the bioassay design (number of animals, number of dilutions, replicates per dilution, dilution spacing, etc.); by the assay acceptance and sample acceptance criteria; and by the statistical analysis (where the primary endpoints are the similarity assessment for each sample and potency estimates for the reference samples). Operating restrictions and bioassay design (intra- and inter-run formulae that result in a *reportable value* for a test material) are usually specified during development and may become a part of the bioassay operating procedure. IP is studied by independent *runs* of the procedure, perhaps using an experimental design that alters those factors that may have an impact on the performance of the procedure. Experiments (including those that implement formalized design of experiments [DOE]) with nested or crossed design structure can reveal important sources of variability in the procedure, as well as ensure a representative estimate of long-term variability. During the validation it is not necessary to employ the format required to achieve the reportable value for a Test sample. A well-designed validation experiment that combines both intra-run and inter-run sources of variability provides estimates of independent components of the bioassay variability. These components can be used to verify or forecast the variability of the bioassay format.

A thorough analysis of the validation data should include graphical and statistical summaries of the validation parameters' results and their conformance to target acceptance criteria. The analysis should follow the specifics of the data-analysis plan outlined in the validation protocol. In most cases, log relative potency should be analyzed in order to satisfy the assumptions of the statistical methods (see section 2.7 *Statistical Considerations, Scale of Analysis,*). Those assumptions include *normality* of the distribution from which the data were sampled and *homogeneity of variability* across the range of results observed in the validation. These assumptions can be explored using graphical techniques such as box plots and probability plots. The assumption of normality can be investigated using statistical tests of normality across a suitably sized collection of historical results. Alternative methods of analysis should be sought when the assumptions can be challenged. *Confidence intervals* should be calculated for the validation parameters, using methods described here and in general chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩.

2.4 Validation Strategies for Bioassay Performance Characteristics

Parameters that should be verified in a bioassay are *relative accuracy, specificity,* IP (which incorporates repeatability), and *range*. Other parameters discussed in general chapter ⟨1225⟩ and ICH Q2(R1) such as detection limit and quantitation limit have not been included because they are usually not relevant to a bioassay that reports relative potency. These may be relevant, however, to the validation of an ancillary assay such as one used to score responders or measure response in conjunction with an *in vivo* potency assay. Likewise linearity is not part of bioassay validation, except as it relates to relative accuracy (dilutional linearity). There follow strategies for addressing bioassay validation parameters.

Relative Accuracy

The *relative accuracy* of a relative potency bioassay is the relationship between measured relative potency and known relative potency. Relative accuracy in bioassay refers to a unit slope (slope = 1) between log measured relative potency and log known relative potency. The most common approach to demonstrating relative accuracy for relative potency bioassays is by construction of target potencies by dilution of the standard material or a Test sample with known potency. This type of study is often referred to as a *dilutional linearity study*. The results from a dilutional linearity study should be assessed using the estimated relative bias at individual levels and via a trend in *relative bias* across levels. The *relative bias* at individual levels is calculated as follows:

$$\text{Relative Bias} = 100 \cdot \left(\frac{\text{Measured Potency}}{\text{Target Potency}} - 1 \right)\%$$

The trend in bias is measured by the estimated slope of log measured potency versus log target potency, which should be held to a target acceptance criterion. If there is no trend in *relative bias* across levels, the estimated *relative bias* at each level can be held to a prespecified target acceptance criterion that has been defined in the validation protocol (see section 3 *A Bioassay Validation Example*).

Specificity—For products or intermediates associated with complex matrices, specificity involves demonstrating lack of interference from matrix components or product-related components that can be expected to be present. This can be assessed via parallel dilution of the Standard with and without a spike addition of the potentially interfering compound. If the curves are similar and the potency conforms to expectations of a Standard-to-Standard comparison, the bioassay is specific against the compound. For these assessments both similarity and potency may be assessed using appropriate equivalence tests.

Specificity may also refer to the capacity of the bioassay to distinguish between different but related biopharmaceutical molecules. An understanding should be sought of the molecule and any related forms, and of opportunities for related molecules to be introduced into the bioassay.

Intermediate Precision—Because of potential influences on the bioassay by factors such as analysts, instruments, or reagent lots, the design of the bioassay validation should include evaluation of these factors. The overall variability from measurements taken under a variety of normal test conditions within one laboratory defines the IP of the bioassay. IP is the ICH and USP term for what is also commonly referred to as inter-run variability. IP measures the influence of factors that will vary over time after

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the bioassay is implemented. These influences are generally unavoidable and include factors like change in personnel (new analysts), receipt of new reagent lots, etc.

When the validation has been planned using multifactor DOE, the impact of each factor can first be explored graphically to establish important contributions to potency variability. The identification of important factors should lead to procedures that seek to control their effects, such as further restrictions on intra-assay operating conditions or strategic qualification procedures on inter-run factors such as analysts, instruments, and reagent lots.

Contributions of validation study factors to the overall IP of the bioassay can be determined by performing a *variance component analysis* on the validation results. *Variance component analysis* is best carried out using a statistical software package that is capable of performing a mixed-model analysis with restricted maximum likelihood estimation (REML).

A variance component analysis yields variance component estimates such as

$$\hat{\sigma}^2_{Intra}$$

and

$$\hat{\sigma}^2_{Inter}$$

corresponding to intra-run and inter-run variation. These can be used to estimate the IP of the bioassay, as well as the variability of the *reportable value* for different bioassay formats (format variability). IP expressed as percent *geometric* coefficient of variation (%GCV) is given by the following formula, in this case using the natural log of the relative potency in the analysis (see section 2.7 *Statistical Considerations, Scale of Analysis*):

$$\text{Intermediate Precision} = 100 \cdot \left(e^{\sqrt{\sigma^2_{Inter} + \sigma^2_{Intra}}} - 1\right)\%$$

The variability of the *reportable value* from testing performed with n replicate sets in each of k runs (*format variability*) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{\sigma^2_{Inter}/k + \sigma^2_{Intra}/(nk)}} - 1\right)\%$$

This formula can be used to determine a testing format suitable for various uses of the bioassay (e.g., release testing and stability evaluation).

Range—The *range* of the bioassay is defined as the true or known potencies for which it has been demonstrated that the analytical procedure has a suitable level of relative accuracy and IP. The range is normally derived from the dilutional linearity study and minimally should cover the product specification range for potency. For stability testing and to minimize having to dilute or concentrate hyper- or hypo-potent Test samples into the bioassay range, there is value in validating the bioassay over a broader range.

2.5 Validation Target Acceptance Criteria

The validation target acceptance criteria should be chosen to minimize the risks inherent in making decisions from bioassay measurements and to be reasonable in terms of the capability of the art. When there is an existing product specification, acceptance criteria can be justified on the basis of the risk that measurements may fall outside of the product specification. Considerations from a process capability (Cp) index can be used to inform bounds on the *relative bias* (RB) and the IP of the bioassay. This chapter uses the following Cpm index:

$$Cpm = \frac{USL - LSL}{6 \cdot \sqrt{\sigma^2_{Product} + RB^2 + \sigma^2_{RA}}}$$

where USL and LSL are the upper and lower release specification, RB is a bound on the degree of relative bias in the bioassay, and

$$\sigma^2_{Product}$$

and

$$\sigma^2_{RA}$$

are target product variance (i.e., lot-to-lot variability) and release assay variance (with associated format) respectively. (See section 3 *A Bioassay Validation Example* for an example of determination of

$$\sigma^2_{RA}$$

and Cpm.) This formulation requires prior knowledge regarding target product variability, or the inclusion of a random selection of lots to estimate this characteristic as part of the validation. Given limited understanding of assay performance, manufacturing

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history, and final specifications during development, this approach may be used simply as a guide for defining validation acceptance criteria.

The choice of a bound on Cpm is a business decision. The proportion of lots that are predicted to be outside their specification limits is a function of Cpm. Some laboratories require process capability corresponding to Cpm greater than or equal to 1.3. This corresponds to approximately a 1 in 10,000 chance that a lot with potency at the center of the specification range will be outside the specification limits.

When specifications have yet to be established for a product, a restriction on *relative bias* or IP can be formulated on the basis of the capability of the art of the bioassay methodology. For example, although chemical assays and immunoassays are often capable of achieving near single digit *percent coefficient of variation* (%CV, or percent relative standard deviation, %RSD), a more liberal restriction might be placed on bioassays, such as animal potency bioassays, that operate with much larger variability (measured as %GCV which can be compared to %CV; see *Appendix 1*). In this case the validation goal might be to *characterize* the method, using the validation results to establish an assay format that is predicted to yield reliable product measurements. A sound justification for target acceptance criteria or use of *characterization* should be included in the validation protocol.

2.6 Assay Maintenance

Once a bioassay has been validated it can be implemented. However, it is important to monitor its behavior over time. This is most easily accomplished by maintaining *statistical process control* (SPC) charts for suitable parameters of the Standard response curve and potency of assay QC samples. The purpose of these charts is to identify at an early stage any shift or drift in the bioassay. If a trend is observed in any SPC chart, the reason for the trend should be identified. If the resolution requires a modification to the bioassay or if a serious modification of the bioassay has occurred for other reasons (for example, a major technology change), the modified bioassay should be revalidated or linked to the original bioassay by an adequately designed bridging study with acceptance criteria that use equivalence testing.

2.7 Statistical Considerations

Several statistical considerations are associated with designing a bioassay validation and analyzing the data. These relate to the properties of bioassay measurements as well as the statistical tools that can be used to summarize and interpret bioassay validation results.

SCALE OF ANALYSIS

The scale of analysis of bioassay validation, where data are the relative potencies of samples in the validation study, must be considered in order to obtain reliable conclusions from the study. This chapter assumes that appropriate methods are already in place to reduce the raw bioassay response data to relative potency (as described in general chapter ⟨1034⟩). Relative potency measurements are typically nearly *log normally distributed.Log normally distributed* measurements are skewed and are characterized by *heterogeneity of variability,* where the standard deviation is proportional to the level of response. The statistical methods outlined in this chapter require that the data be symmetric, approximating a normal distribution, but some of the procedures require *homogeneity of variability* in measurements across the potency range. Typically, analysis of potency after log *transformation* generates data that more closely fulfill both of these requirements. The base of the log transformation does not matter as long as a consistent base is maintained throughout the analysis. Thus, for example, if the natural log (log to the base e) is used to transform relative potency measurements, summary results are converted back to the bioassay scale utilizing base e.

The distribution of potency measurements should be assessed as part of bioassay development (as described in ⟨1032⟩). If it is determined that potency measurements are normally distributed, the validation can be carried out using methods described in the general chapter *Validation of Compendial Procedures* ⟨1225⟩.

As a consequence of the usual (for parallel-line assays) log transformation of relative potency measurements, there are advantages if the levels selected for the validation study are evenly spaced on the log scale. An example with five levels would be 0.50, 0.71, 1.00, 1.41, and 2.00. Intermediate levels are obtained as the *geometric mean* of two adjacent levels. Thus for example, the mid-level between 0.50 and 1.0 is derived as follows:

$$GM=\sqrt{0.50\cdot 1.0}=0.71$$

Likewise, summary measures of the validation are influenced by the log normal scale. Predicted response should be reported as the *geometric mean* of individual relative potency measurements, and variability expressed as %GCV. GCV is calculated as the anti-log of the standard deviation, S_{log}, of log transformed relative potency measurements. The formula is given by:

$$GCV = \text{antilog}(S_{log}) - 1$$

Variability is expressed as GCV rather than RSD of the log normal distribution in order to preserve continuity using the log transformation (see additional discussion in the *Appendix 1* to this chapter). Intervals that might be calculated from GCV will be consistent with intervals calculated from mean and standard deviation of log transformed data. *Table 1* presents an example of the calculation of geometric mean (GM) and associated RB, with %GCV for a series of relative potency measurements performed on samples tested at the 1.00 level. The log base e is used in the illustration.

Table 1. Illustration of calculations of GM and %GCV

RP[1]	ln RP	
1.1299	0.1221	

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Table 1. Illustration of calculations of GM and %GCV *(continued)*

RP[1]	ln RP	
0.9261	−0.0768	
1.1299	0.1221	
1.0143	0.0142	
1.0027	0.0027	
1.0316	0.0311	
1.1321	0.1241	
1.0499	0.0487	
Average	0.0485	GM = 1.0497
		RB = 4.97%
SD	0.0715	%GCV = 7.4%

[1] Relative potency (RP) is the geometric mean of duplicate potencies measured in the eight runs of the example given in *Table 4*.

Here the GM of the relative potency measurements is calculated as the anti-log of the average log relative potency measurements and then expressed as relative bias, the percent deviation from the target potency:

$$GM = e^{Average} = e^{0.0485} = 1.0497$$

$$RB = 100 \cdot \left(\frac{GM}{Target} - 1\right)\% = 100 \cdot \left(\frac{1.0497}{1.00} - 1\right)\% = 4.97\%$$

and the percent *geometric coefficient of variation* (%GCV) is calculated as:

$$\%GCV = 100 \cdot (e^{SD} - 1)\% = 100 \cdot (e^{0.0715} - 1)\% = 7.4\%$$

Note that the %GCV calculated for this illustration is not equal to the IP determined in the bioassay validation example for the 1.00 level (8.5%); see *Table 6*. This illustration utilizes the average of within-run replicates, while the IP in the validation example represents the variability of individual replicates.

Reporting Validation Results Using Confidence Intervals—Estimates of bioassay validation parameters should be presented as a *point estimate* together with a *confidence interval.* A *point estimate* is the numerical value obtained for the parameter, such as the GM or %GCV. A *confidence interval's* most common interpretation is as the likely range of the true value of the parameter. The previous example determines a 90% *confidence interval* for average log relative potency, CI_{ln}, as follows:

$$CI_{ln} = Average \pm t_{df} \cdot SD/\sqrt{n}$$
$$= 0.0485 \pm 1.89 \cdot 0.0715/\sqrt{8} = (0.0007, 0.0963)$$

For percent relative bias this is:

$$CI_{RB} = 100 \cdot \left(\frac{e^{0.0007}}{1.00} - 1\right)\%, 100 \cdot \left(\frac{e^{0.0963}}{1.00} - 1\right)\% = (0.07\%, 10.1\%)$$

The statistical constant (1.89) is from a t-table, with degrees of freedom (df) equal to the number of measurements minus one (df = 8 − 1 = 7). A confidence interval for IP or format variability can be formulated using methods for variance components; these methods are not covered in this general chapter.

Assessing Conformance to Acceptance Criteria—Bioassay validation results are compared to target acceptance criteria in order to demonstrate that the bioassay is fit for use. The process of establishing conformance of validation parameters to validation acceptance criteria should not be confused with establishing conformance of relative potency measurements to product specifications. Product specifications should inform the process of setting validation acceptance criteria.

A common practice is to apply acceptance criteria to the estimated validation parameter. This does not account, however, for the uncertainty in the estimated validation parameter. A solution is to hold the *confidence interval* on the validation parameter to the acceptance criterion. This is a standard statistical approach used to demonstrate conformance to expectation and is called an *equivalence test.* It should not be confused with the practice of performing a significance test, such as a t-test, which seeks to establish a difference from some target value (e.g., 0% relative bias). A significance test associated with a P-value > 0.05 (equivalent to a confidence interval that includes the target value for the parameter) indicates that there is insufficient evidence to conclude that the parameter is different from the target value. This is not the same as concluding that the parameter conforms to its target value. The study design may have too few *replicates,* or the validation data may be too variable to discover a meaningful difference from target. Additionally, a significance test may detect a small deviation from target that is of negligible importance. These scenarios are illustrated in *Figure 1*.

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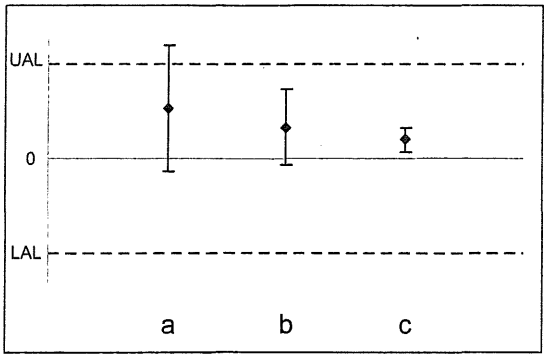

Figure 1. Use of confidence intervals to establish that validation results conform to an acceptance criterion.

The solid horizontal line represents the target value (perhaps 0% relative bias), and the dashed lines form the lower (LAL) and upper (UAL) acceptance limits. In scenario a, the confidence bound includes the target, and thus one could conclude there is insufficient evidence to conclude a difference from target (the significance test approach). However, although the *point estimate* (the solid diamond) falls within the acceptance range, the interval extends outside the range, which signifies that the true relative bias may be outside the acceptable range. In scenario b, the interval falls within the acceptance range, signifying conformance to the acceptance criterion. The interval in scenario c also falls within the acceptance range but excludes the target. Thus, for scenario c, although the difference of the point estimate from the target is statistically significant, c is acceptable because the confidence interval falls within the target acceptance limits.

Using the 90% confidence interval calculated previously, we can establish whether the bioassay has acceptable relative bias at the 1.00 level compared to a target acceptance criterion of no greater than +12%, for example. Because the 90% confidence interval for percent relative bias (0.07%, 10.1%) falls within the interval (100*[(1/1.12) – 1]%, 100*[(1.12/1) – 1]%) = (– 11%, 12%), we conclude that there is acceptable relative bias at the 1.00 level. Note that a 90% confidence interval is used in an equivalence test rather than a conventional 95% confidence interval. This is common practice and is the same as the *two one-sided tests* (TOST) approach used in pharmaceutical bioequivalence testing.

Risks in Decision-Making and Number of Validation Runs—The application of statistical tests, including the assessment of conformance of a validation parameter to its acceptance criteria, involves risks. One risk is that the parameter does not meet its acceptance criterion although the property associated with that parameter is satisfactory; another, the converse, is that the parameter meets its acceptance criterion although the parameter is truly unsatisfactory. A consideration related to these risks is sample size.

The two types of risk can be simultaneously controlled via strategic design, including choice of the number of *runs* that will be conducted in the validation. Specifically, the minimum number of *runs* needed to establish conformance to an acceptance criterion for relative bias is given by:

$$n \geq \frac{\left(t_{\alpha,df} + t_{\beta/2,df}\right)^2 \hat{\sigma}_{IP}^2}{\theta^2}$$

where $t_{\alpha,df}$ and $t_{\beta,df}$ are distributional points from a Student's t-distribution; α and β are the one-sided type I and type II errors, and represent the risks associated with drawing the wrong conclusion in the validation; df is the degrees of freedom associated with the study design (usually n – 1);

$$\hat{\sigma}_{IP}^2$$

is a preliminary estimate of IP; and θ is the acceptable deviation (target acceptance criterion).

For example, if the acceptance criterion for relative bias is ± 0.11 log (i.e., $\theta = 0.11$), the bioassay variability is

$$\hat{\sigma}_{IP} = 0.076 \text{ log}$$

and $\alpha = \beta = 0.05$,

$$n \geq \frac{(1.89 + 2.36)^2 \, 0.076^2}{0.11^2} \approx 8 \text{ runs}$$

Note that this formulation of sample size assumes no intrinsic bias in the bioassay. A more conservative solution includes some nonzero bias in the determination of a sample size. This results in a greater sample size to offset the impact of the bias on the conclusions of the validation. In the current example the sample size increases to 10 runs if one assumes an intrinsic bias equal to 2%. Note also that this calculation represents a recursive solution (because the degrees of freedom depend on n) requiring statistical software or an algorithm that employs iterative methodology.

Note further that the selection of α and β should be justified on the basis of the corresponding risks of drawing the wrong conclusion from the validation.

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Modeling Validation Results Using Mixed Effects Models—Many analyses associated with bioassay validation must account for multiple design factors such as *fixed effects* (e.g., potency level), as well as *random effects* (e.g., analyst, run, and replicate). Statistical models composed of both *fixed* and *random effects* are called *mixed effects models* and usually require sophisticated statistical software for analysis. The results of the analysis may be summarized in an *analysis of variance* (ANOVA) table or a table of variance component estimates. The primary goal of the analysis is to estimate critical parameters rather than establish the significance of an effect. The modeling output provides parameter estimates together with their *standard errors* of estimates that can be utilized to establish conformance of a validation parameter to its acceptance criterion. Thus the average *relative bias* at each level is obtained as a portion of the analysis together with its associated variability. These compose a *confidence interval* that is compared to the acceptance criterion as described above. If variances across levels can be pooled, statistical modeling can also determine the overall *relative bias* and IP by combining information across levels performed in the validation. Similarly, mixed effects models can be used to obtain variance components for validation study factors and to combine results across validation study samples and levels.

Statistical Design—Statistical designs, such as multifactor DOE or *nesting,* can be used to organize assay and runs in a bioassay validation. It is useful to incorporate factors that are believed to influence the bioassay response and that vary during long-term use of the procedure into these designs. Using these methods of design, the sources of variability may be characterized and a strategic test plan to manage the variability of the bioassay may be developed.

Table 2 shows an example of a multifactor DOE that incorporates multiple analysts, multiple cell culture preparations, and multiple reagent lots into the validation plan.

Table 2. Example of a Multifactor DOE with 3 Factors

Run	Analyst	Cell Prep	Reagent Lot
1	1	1	1
2	1	1	2
3	1	2	1
4	1	2	2
5	2	1	1
6	2	1	2
7	2	2	1
8	2	2	2

In this design each analyst performs the bioassay with both cell preparations and both reagent lots. This is an example of a *full factorial* design because all combinations of the factors are performed in the validation study. To reduce the number of runs in the study, *fractional factorial* designs may be employed when more than three factors have been identified. For example, if it is practical for an analyst to perform four assays in a run, a split-unit design could be used with analysts as the whole-plot factor and cell preparation and reagent lot as sub-plot factors. Unlike screening experiments, the validation design should incorporate as many factors at as many levels as possible in order to obtain a representative estimate of IP. More than two levels of a factor should be employed in the design whenever possible. This may be accomplished in a less structured manner, without regard to strict factorial layout. Validation runs should be randomized whenever possible to mitigate the potential influences of run order or time.

Figure 2 illustrates an example of a validation using *nesting* (replicates nested within plate, plate nested within analyst).

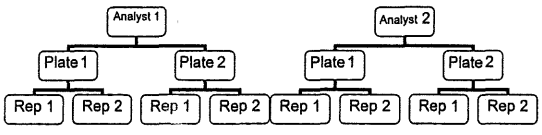

Figure 2. Example of a nested design using two analysts.

For both of these types of design as well as combinations of the two, components of variability can be estimated from the validation results. These components of variability can be used to identify significant sources of variability as well as to derive a bioassay *format* that meets the procedure's requirements for precision. It should be noted that significant sources of variability may have been identified during bioassay development. In this case the validation should confirm both the impact of these factors and the assay format that meets the requirement for precision.

Significant Figures—The number of significant figures in a reported result from a bioassay is related to the latter's precision. In general, a bioassay with %GCV between 2% and 20% will support two significant figures. The number of significant figures should not be confused with the number of decimal places—reported values equal to 1.2 and 0.12 have the same number (two) of significant figures. This standard of rounding is appropriate for log scaled measurements that have constant variation on the log scale and proportional rather than additive variability on the original scale (or the scale commonly used for interpretation). Note that rounding occurs at the end of a series of calculations when the final measurement is reported and used for decision making such as conformance to specifications. Thus if the final measurement is a reportable value from multiple assays, rounding should not occur prior to determination of the reportable value. Likewise, specifications should be stated with the appropriate number of significant figures.

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3. A BIOASSAY VALIDATION EXAMPLE

An example illustrates the principles described in this chapter. The bioassay will be used to support a specification range of 0.71 to 1.41 for the product. Using the Cpm described in section 2.5 *Validation Target Acceptance Criteria*, a table is derived showing the projected rate of OOS results for various restrictions on RB and IP. Cpm is calculated on the basis of the variability of a reportable value using three independent runs of the bioassay (see discussion of format variability, above). Product variability is assumed to be equal to 0 in the calculations. The laboratory may wish to include target product variability. An estimate of target product variability can be obtained from data from a product, for example, manufactured by a similar process.

Table 3. Cpm and Probability of OOS for Various Restrictions on RB and IP

LSL-USL	IP (%)	RB (%)	Cpm	Prob(OOS) (%)
0.71–1.41	20	20	0.54	10.5
0.71–1.41	8	12	0.94	0.48
0.71–1.41	10	5	1.55	0.0003

The calculation is illustrated for IP equal to 8% and relative bias equal to 12% (n = 3 runs):

$$Cpm = \frac{\ln(1.41) - \ln(0.71)}{6 \cdot \sqrt{[\ln(1.08)]^2/3 + [\ln(1.12)]^2}} = 0.94$$

$$Prob(OOS) = 2 \cdot \Phi(-3 \cdot 0.94) = 0.0048\ (0.48\%),$$

where Φ represents the standard normal cumulative distribution function.

From *Table 3*, acceptable performance (less than 1% chance of obtaining an OOS result due to bias and variability of the bioassay) can be expected if the IP is ≤8% and relative bias is ≤12%. The sample size formula given in section 2.7 *Statistical Considerations, Risks in Decision-Making and Number of Validation Runs* can be used to derive the number of runs required to establish conformance to an acceptance criterion for relative bias equal to 12% (using $\%GCV_{IP}$ = 8%; $\alpha = \beta = 0.05$):

$$n \geq \frac{(1.89 + 2.36)^2 \cdot [\ln(1.08)]^2}{[\ln(1.12)]^2} \approx 8 \text{ runs}$$

Thus eight runs would be needed in order to have a 95% chance of passing the target acceptance criterion for relative bias if the true relative bias is zero. Note that the calculation of sample size assumes that a singlet of the validation samples will be performed in each validation run. The use of multiple replication sets and/or multiple assays will provide valuable information that allows separate estimates for intra-run and inter-run variability, and will decrease the risk of failing to meet the validation target acceptance criteria.

Five levels of the target analyte are studied in the validation: 0.50, 0.71, 1.00, 1.41, and 2.00. Two runs at each level are generated by two trained analysts using two media lots. Other factors may be considered and incorporated into the design using a fractional factorial layout. The laboratory should strive to design the validation with as many levels of each factor as possible in order to best model the long-term performance of the bioassay. In this example each analyst performs two runs at each level using each media lot. A run consists of a full dilution series of the Standard as described in the bioassay's operating procedure, together with two independent dilution series of the Test sample. This yields duplicate measurements of relative potency in each run; see *Table 4* for all relative potency observations. Note that the two potency estimates at each level of potency in a run are not independent due to common analysts and media lots.

Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot

Media Lot/Analyst	1/1		1/2		2/1		2/2	
Run	1	2	1	2	1	2	1	2
0.50	0.5215	0.4532	0.5667	0.5054	0.5222	0.5179	0.5314	0.5112
0.50	0.5026	0.4497	0.5581	0.5350	0.5017	0.5077	0.5411	0.5488
0.71	0.7558	0.6689	0.6843	0.7050	0.6991	0.7463	0.6928	0.7400
0.71	0.7082	0.6182	0.8217	0.7143	0.6421	0.6877	0.7688	0.7399
1.00	1.1052	0.9774	1.1527	0.9901	1.0890	1.0314	1.1459	1.0273
1.00	1.1551	0.8774	1.1074	1.0391	0.9233	1.0318	1.1184	1.0730
1.41	1.5220	1.2811	1.5262	1.4476	1.4199	1.3471	1.4662	1.5035
1.41	1.5164	1.3285	1.5584	1.4184	1.4025	1.4255	1.5495	1.5422
2.00	2.3529	1.8883	2.3501	2.2906	2.2402	2.1364	2.3711	2.0420

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Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot *(continued)*

Media Lot/Analyst	1/1		1/2		2/1		2/2	
2.00	2.2307	1.9813	2.4013	2.1725	2.0966	2.1497	2.1708	2.3126

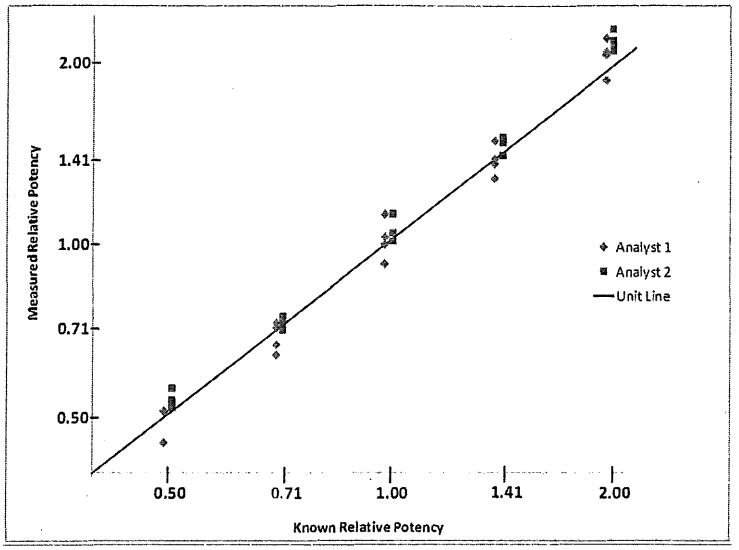

Figure 3. A plot of the validation results versus the sample levels.

A plot is used to reveal irregularities in the experimental results. In particular, a properly prepared plot can reveal a failure in agreement of validation results with validation levels, as well as *heterogeneity of variability* across levels (see discussion of the log transformation in section 2.7 *Statistical Considerations*). The example plot in *Figure 3* includes the unit line (line with slope equal to 1, passing through the origin). The analyst 1 and analyst 2 data are deliberately offset with respect to the expected potency to allow clear visualization and comparison of the data sets from each analyst.

A formal analysis of the validation data might be undertaken in the following steps: (1) an assessment of variability (IP) should precede an assessment of relative accuracy or specificity in order to establish conformance to the assumption that variances across sample levels can be pooled; and (2) *relative accuracy* is assessed either at separate levels or by a combined analysis, depending on how well the data across levels can be pooled. These steps are demonstrated using the example validation data, along with some details of the calculations for illustrative purposes. Note that the calculations illustrated in the following sections are appropriate only with a balanced dataset. Imbalanced designs or datasets with missing relative potency measurements should be analyzed using a mixed model analysis with restricted maximum likelihood estimation (REML).

3.1 Intermediate Precision

Data at each level can be analyzed using *variance component analysis*. With balanced data, as in this example, variance components can be determined from a standard one-way ANOVA. An example of the calculation performed at a single level (0.50) is presented in *Table 5*.

Table 5. Variance Component Analysis Performed on Log Relative Potency Measurements at the 0.5 Level

Source	df	Sum of Squares	Mean Square	Expected Mean Square
Run	7	0.055317	0.007902	Var(Error) + 2 Var(Run)
Error	8	0.006130	0.000766	Var(Error)
Corrected total	15	0.061447		
Variance Component Estimates				
Var(Run) = 0.003568				
Var(Error) = 0.000766				

The top of the table represents a standard ANOVA analysis. Analyst and media lot have not been included because of the small number of levels (2 levels) for each factor. The factor "Run" in this analysis represents the combined runs across the analyst by media lot combinations. The Expected Mean Square is the linear combination of variance components that generates the measured *mean square* for each source. The variance component estimates are derived by solving the equation "Expected Mean

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Square = Mean Square" for each component. To start, the *mean square* for Error estimates Var(Error), the within-run component of variability, is

$$\text{Var(Error)} = \text{MS(Error)} = 0.000766$$

The between-run component of variability, Var(Run), is subsequently calculated by setting the mean square for Run to the mathematical expression for the expected mean square, then solving the equation for Var(Run) as follows:

$$\text{MS(Run)} = \text{Var(Error)} + 2 \cdot \text{Var(Run)}$$

$$\text{Var(Run)} = \frac{\text{MS(Run)} - \text{MS(Error)}}{2}$$

$$= \frac{0.007902 - 0.000766}{2} = 0.003568$$

These *variance component estimates* are combined to establish the overall IP of the bioassay at 0.50:

$$\text{IP} = 100 \cdot \left(e^{\sqrt{\text{Var(Run)}+\text{Var(Error)}}} - 1\right)\%$$

$$= 100 \cdot \left(e^{\sqrt{0.003568+0.000766}} - 1\right)\% = 6.8\%$$

The same analysis was performed at each level of the validation, and is presented in *Table 6*.

Table 6. Variance Component Estimates and Overall Variability for Each Validation Level and the Average

	Level					
Component	0.50	0.71	1.00	1.41	2.00	Average
Var(Run)	0.003568	0.000648	0.003639	0.003135	0.002623	0.002723
Var(Error)	0.000766	0.004303	0.002954	0.000577	0.002258	0.002172
Overall	6.8%	7.3%	8.5%	6.3%	7.2%	7.2%

A combined analysis can be performed if the variance components are similar across levels. Typically a heuristic method is used for this assessment. One might hold the ratio of the maximum variance to the minimum variance to no greater than 10 (10 is used because of the limited number of runs performed in the validation). Here the ratios associated with the between-run variance component, 0.003639/0.000648 = 5.6, and the within-run component, 0.004303/0.000577 = 7.5, meet the 10-fold criterion. Had the ratio exceeded 10 and if this was due to excess variability in one or the other of the extremes in the levels tested, that extreme would be eliminated from further analysis and the range would be limited to exclude that level.

The analysis might proceed using statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis should account for any imbalance in the design, random effects such as analyst and media lot, and fixed effects such as level (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*). Variance components can be determined for analyst and media lot separately in order to characterize their contributions to the overall variability of the bioassay.

In the example, variance components can be averaged across levels to report the IP of the bioassay. This method of combining estimates is exact only if a balanced design has been employed in the validation (i.e., the same replication strategy at each level). A balanced design was employed for the example validation, so the IP can be reported as 7.2% GCV.

Because of the recommendation to report validation results with some measure of uncertainty, a one-sided 95% upper confidence bound can be calculated for the IP of the bioassay. The literature contains methods for calculating confidence bounds for variance components. The upper bound on IP for the bioassay example is 11.8% GCV. The upper confidence bound was not calculated at each level separately because of the limited data at an individual level relative to the overall study design.

3.2 Relative Accuracy

The analysis might proceed with an assessment of relative accuracy at each level. *Table 7* shows the average and 90% confidence interval of validation results in the log scale, as well as corresponding potency and relative bias.

Table 7. Average Potency and Relative Bias at Individual Levels

		Log Potency		Potency		Relative Bias	
Level	n [a]	Average	(90% CI)	Average	(90% CI)	Average	(90% CI)
0.50	8	–0.6613	(–0.7034, –0.6192)	0.52	(0.49, 0.54)	3.23%	(–1.02, 7.67)
0.71	8	–0.3419	(–0.3773, –0.3064)	0.71	(0.69, 0.74)	0.06%	(–3.42, 3.67)
1.00[b]	8	0.0485	(0.0006, 0.0964)	1.05	(1.00, 1.10)	4.97%	(0.06, 10.12)
1.41	8	0.3723	(0.3331, 0.4115)	1.45	(1.40, 1.51)	2.91%	(–1.04, 7.03)

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Table 7. Average Potency and Relative Bias at Individual Levels *(continued)*

		Log Potency		Potency		Relative Bias	
Level	n [a]	Average	(90% CI)	Average	(90% CI)	Average	(90% CI)
2.00	8	0.7859	(0.7449, 0.8269)	2.19	(2.11, 2.29)	9.72%	(5.31, 14.32)

[a] Analysis performed on averages of duplicates from each run.
[b] Calculation illustrated in section 2.7 *Statistical Considerations, Scale of Analysis.*

The analysis has been performed on the average of the duplicates from each run (n = 8 runs) because duplicate measurements are correlated within a run by shared IP factors (analyst, media lot, and run in this case). A plot of relative bias versus level can be used to examine patterns in the experimental results and to establish conformance to the target acceptance criterion for relative bias (12%).

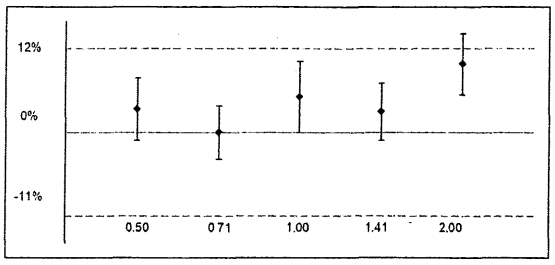

Figure 4. Plot of 90% confidence intervals for relative bias versus the acceptance criterion. Note lower acceptance criterion is equal to $100 \cdot [(1/1.12) - 1] = -11\%$.

Figure 4 shows an average positive bias across sample levels (i.e., the average relative bias is positive at all levels). This consistency is due in part to the lack of independence of bioassay results across levels. In addition there does not appear to be a trend in relative bias across levels. The latter would indicate that a comparison of samples with different measured relative potency (such as stability samples) is biased, resulting perhaps in an erroneous conclusion. Trend analysis can be performed using a regression of log relative potency versus log level. Introduction during the development of the bioassay validation protocol of an acceptance criterion on a trend in relative accuracy across the range can be considered.

After establishing that there is no meaningful trend across levels, the analysis proceeds with an assessment of the relative accuracy at each level. The bioassay has acceptable relative bias at levels from 0.50 to 1.41, yielding 90% confidence bounds (equivalent to a *two one-sided t-test*) that fall within the acceptance region of –11% to 12% relative bias. The 90% confidence interval at 2.0 falls outside the acceptance region, indicating that the relative bias may exceed 12%.

A combined analysis can be performed utilizing statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis accurately accounts for the validation study design. The analysis also accommodates *random effects* such as analyst, media lot, and run (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*).

3.3 Range

The conclusions derived from the assessment of IP and *relative accuracy* can be used to establish the bioassay's range that demonstrates satisfactory performance. Based on the acceptance criterion for IP equal to 8% GCV (see *Table 6*) and for relative bias equal to 12% (see *Table 7*), the range of the bioassay is 0.50 to 1.41. In this range, level 1.0 has a slightly higher than acceptable estimate of IP (8.5% versus the target acceptance criterion ≤8.0%), which may be due to the variability of the estimate that results from a small dataset. Because of this and other results in *Table 6*, one may conclude that satisfactory IP was demonstrated across the range.

3.4 Use of Validation Results for Bioassay Characterization

When the study has been performed to estimate the characteristics of the bioassay (characterization), the *variance component estimates* can also be used to predict the variability for different bioassay *formats* and thereby can determine a format that has a desired level of precision. The predicted variability for k independent *runs,* with n individual dilution series of the test preparation within a run, is given by the following formula for *format variability:*

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{\text{Var(Run)}/k + \text{Var(Error)}/(nk)}} - 1\right)$$

Using estimates of intra-run and inter-run variance components from *Table 6* [Var(Run) = 0.002723 and Var(Error) = 0.002172] , if the bioassay is performed in three independent *runs,* the predicted variability of the *reportable value* (geometric mean of the relative potency results) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{0.002723/3 + 0.002172/(1 \cdot 3)}} - 1\right) = 4.1\%$$

This calculation can be expanded to include various combinations of *runs* and *minimal* sets (assuming that the numbers of samples, dilutions, and replicates in the minimal sets are held constant) within *runs* as shown in *Table 8.*

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Table 8. Format Variability for Different Combinations of Number of Runs (k) and Number of Minimal Sets within Run (n)

	Number of Runs (k)			
Reps (n)	1	2	3	6
1	7.2%	5.1%	4.1%	2.9%
2	6.4%	4.5%	3.6%	2.6%
3	6.0%	4.2%	3.4%	2.4%
6	5.7%	4.0%	3.3%	2.3%

Clearly the most effective means of reducing the variability of the *reportable value* (the geometric mean potency across runs and minimal sets) is by independent runs of the bioassay procedure. In addition, confidence bounds on the variance components used to derive IP can be utilized to establish the bioassay's format variability.

Significant sources of variability must be incorporated into runs in order to effect variance reduction. A more thorough analysis of the bioassay validation example would include analyst and media lot as factors in the statistical model. Variance component estimates obtained from such an analysis are presented in *Table 9*.

Table 9. REML Estimates of Variance Components Associated with Analyst, Media Lot, and Run

Variance	Component Estimate
Var(Media Lot)	0.0000
Var(Analyst)	0.0014
Var(Analyst*Media Lot)	0.0000
Var(Run (Analyst*Media Lot))	0.0019
Var(Error)	0.0022

Identification of analyst as a significant bioassay factor should ideally be addressed during bioassay development. Nonetheless the laboratory may choose to address the apparent contribution of analyst-to-analyst variability through improved training or by using multiple analysts in formatting the assay for routine performance of the bioassay.

Estimates of intra-run and inter-run variability can also be used to determine the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. For k runs, with n minimal sets within each run, using an approximate two-sided critical value from the standard normal distribution with z = 2, the critical fold difference between reportable values for two samples that are tested in the same runs of the bioassay is given by:

$$\text{Critical Fold Difference} = e^{2 \cdot \sqrt{\text{Var(Run)}/k + \text{Var(Error)}/(nk)}}$$

When samples have been tested in different runs of the bioassay (such as long-term stability samples), the critical fold difference is given by (assuming the same format is used to test the two series of samples):

$$\text{Critical Fold Difference} = e^{2 \cdot \sqrt{2 \cdot [\text{Var(Run)}/k + \text{Var(Error)}/(nk)]}}$$

For comparison of samples the laboratory can choose a design (bioassay format) that has suitable precision to detect a practically meaningful fold difference between samples.

3.5 Confirmation of Intermediate Precision and Revalidation

The estimate of IP from the validation is highly uncertain because of the small number of runs performed. After the laboratory gains suitable experience with the bioassay, the estimate can be confirmed or updated by analysis of control sample measurements such as the variability of a positive control. This analysis can be done with the control prepared and tested like a Test sample (i.e., same or similar dilution series and replication strategy). This assessment should be made after sufficient assays have been performed to obtain an alternative estimate of the bioassay's intermediate precision, including implementation of changes (e.g., different analysts, different key reagent lots, and different cell preparations) associated with the standardized assay protocol. The reported IP of the bioassay should be modified as an amendment to the validation report if the assessment reveals a substantial disparity of results.

The bioassay should be revalidated whenever a substantial change is made to the method. This includes but is not limited to a change in technology or a change in readout. The revalidation may consist of a complete re-enactment of the bioassay validation or a bridging study that compares the current and the modified methods.

APPENDICES

Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables

Two assumptions of common statistical procedures, such as ANOVA or confidence interval estimation, are (1) the variation in the bioassay response about its mean is normally distributed and (2) the standard deviation of the observed response values

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is constant over the range of responses that are of interest. Such responses are said to have a "normal distribution" and an "additive error structure". When these two conditions are not met, it may be useful to consider a transformation before using common statistical procedures.

The variation in bioassay responses is often found to be non-normal (skewed toward higher values) with a standard deviation approximately proportional (or nearly so) to the mean response. Such responses often have a "multiplicative error structure" and follow a "log normal distribution" with a percent coefficient of variation (%CV) that is constant across the response range of interest. In such cases, a log transformation of the bioassay response will be found to be approximately normal with a nearly constant standard deviation over the response range. After log transformation, then, the two assumptions are met, and common statistical procedures can be performed on the log transformed response. The following discussion presumes a log normal distribution for the bioassay response.

We refer to an observed bioassay response value, X, as being on the "original scale of measurement" and to the log transformed response, Y = log(X), as being on the "log transformed scale". Although common statistical procedures may be appropriate only on the log transformed scale, we can summarize bioassay response results by estimating measures of location (e.g., mean or median), measures of spread (e.g., standard deviation), or confidence intervals on either scale of measurement, as long as the scale being used is indicated. The %CV is useful on the original scale where it is constant over the response range. For the same reason, the standard deviation (SD) is relevant on the log transformed scale. There may be advantages to reporting statistical summaries on the basis of the log transformed (Y) scale. However, it is often informative to back transform the reported measures to the original scale of measurement (X).

For any given value of X, there is only one unique value of Y = log(X), and vice versa. Similarly for measures of location and spread, there is a unique one-to-one correspondence between measures of location and spread obtained on the original and log transformed scales. Further, just as there is a simple relationship between X and Y = log(X), there are relatively simple relationships that allow conversion between the corresponding measures on each scale, as indicated in *Table A-1* below. In the table, "Average" and "SD", wherever they appear, refer to measures calculated on the log transformed (Y) scale.

Table A-1. Comparison of Measures of Location and Spread

Measure		Scale of Measurement	
		Log Transformed (Y)	Original (X)
Location		Mean (average)	Geometric mean (GM) $= \sqrt[n]{\prod_{i=1}^{n} x_i} = e^{\text{Average}}$
Spread		Standard deviation (SD)	Geometric standard deviation (GSD) $= e^{SD}$
Confidence intervals (k is an appropriate constant based on the t-distribution or large sample z approximation)	Lower	Average $-$ k $\cdot$ SD/$\sqrt{n}$	GM/GSD$^{k/\sqrt{n}}$
	Upper	Average + k $\cdot$ SD/$\sqrt{n}$	GM $\cdot$ GSD$^{k/\sqrt{n}}$
	Size	Width (upper $-$ lower) $= 2 \cdot k \cdot SD/\sqrt{n}$	Ratio(upper/lower) $=$ GSD$^{2k/\sqrt{n}}$
Percent coefficient of variation (%CV)		%GCV $= 100 \cdot (GSD - 1)$	%CV $= 100\sqrt{e^{SD^2} - 1} \approx$ %GCV

The geometric mean (GM) should not be misinterpreted as an estimate of the mean of the original scale (X) variable, but is instead an estimate of the median of X. The median is a more appropriate measure of location for variables with skewed error distributions such as the log normal, as well as symmetric error distributions where the median is equal to the mean.

Similarly, the geometric standard deviation (GSD) should not be misinterpreted as the standard deviation of the original scale (X) variable. GSD is, however, a useful multiplicative factor for obtaining confidence intervals on the original (X) scale that correspond to those on the log transformed (Y) scale, as shown in the above table. A GSD of 1 corresponds to no variation (SD of Y = 0). The ratio of the Upper to the Lower confidence bounds, on the untransformed (X) scale, will be equal to GSD$^{2k/\sqrt{n}}$, as can be seen from *Table A-1*.

The geometric coefficient of variation (%GCV) approximates the %CV on the original (X) scale when the %CV is below 20%. It is important not to confuse these different measures of spread. The %GCV is a measure relevant to the log transformed (Y) scale, and the %CV is a measure relevant to the original (X) scale. Depending on the preferred frame of reference, either or both measures may be useful.

Appendix 2: Information Sources

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Appendix 3: Additional Sources of Information

Additional information and alternative methods can be found in the references listed below.

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⟨1034⟩ ANALYSIS OF BIOLOGICAL ASSAYS

1. INTRODUCTION

Although advances in chemical characterization have reduced the reliance on bioassays for many products, bioassays are still essential for the determination of potency and the assurance of activity of many proteins, vaccines, complex mixtures, and products for cell and gene therapy, as well as for their role in monitoring the stability of biological products. The intended scope of general chapter *Analysis of Biological Assays* ⟨1034⟩ includes guidance for the analysis of results both of bioassays described in the *United States Pharmacopeia* (*USP*), and of non-USP bioassays that seek to conform to the qualities of bioassay analysis recommended by USP. Note the emphasis on analysis—design and validation are addressed in complementary chapters (*Development and Design of Bioassays* ⟨1032⟩ and *Biological Assay Validation* ⟨1033⟩, respectively).

Topics addressed in ⟨1034⟩ include statistical concepts and methods of analysis for the calculation of potency and confidence intervals for a variety of relative potency bioassays, including those referenced in *USP*. Chapter ⟨1034⟩ is intended for use primarily by those who do not have extensive training or experience in statistics and by statisticians who are not experienced in the analysis of bioassays. Sections that are primarily conceptual require only minimal statistics background. Most of the chapter and all the methods sections require that the nonstatistician be comfortable with statistics at least at the level of *USP* general chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and with linear regression. Most of sections 3.4 *Nonlinear Models for Quantitative Response* and 3.6 *Dichotomous (Quantal) Assays* require more extensive statistics background and thus are intended primarily for statisticians. In addition, ⟨1034⟩ introduces selected complex methods, the implementation of which requires the guidance of an experienced statistician.

Approaches in ⟨1034⟩ are recommended, recognizing the possibility that alternative procedures may be employed. Additionally, the information in ⟨1034⟩ is presented assuming that computers and suitable software will be used for data analysis. This view does not relieve the analyst of responsibility for the consequences of choices pertaining to bioassay design and analysis.

2. OVERVIEW OF ANALYSIS OF BIOASSAY DATA

Following is a set of steps that will help guide the analysis of a bioassay. This section presumes that decisions were made following a similar set of steps during development, checked during validation, and then not required routinely. Those steps and decisions are covered in general information chapter *Design and Development of Biological Assays* ⟨1032⟩. Section 3 *Analysis Models* provides details for the various models considered.

1. As a part of the chosen analysis, select the subset of data to be used in the determination of the relative potency using the prespecified scheme. Exclude only data known to result from technical problems such as contaminated wells, non-monotonic concentration–response curves, etc.
2. Fit the statistical model for detection of potential outliers, as chosen during development, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.
3. Determine which potential outliers are to be removed and fit the model to be used for suitability assessment. Usually, an investigation of outlier cause takes place before outlier removal. Some assay systems can make use of a statistical (noninvestigative) outlier removal rule, but removal on this basis should be rare. One approach to "rare" is to choose the outlier rule so that the expected number of false positive outlier identifications is no more than one; e.g., use a 1%

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test if the sample size is about 100. If a large number of outliers are found above that expected from the rule used, that calls into question the assay.

4. Assess system suitability. System suitability assesses whether the assay Standard preparation and any controls behaved in a manner consistent with past performance of the assay. If an assay (or a run) fails system suitability, the entire assay (or run) is discarded and no results are reported other than that the assay (or run) failed. Assessment of system suitability usually includes adequacy of the fit of the model used to assess similarity. For linear models, adequacy of the model may include assessment of the linearity of the Standard curve. If the suitability criterion for linearity of the Standard is not met, the exclusion of one or more extreme concentrations may result in the criterion being met. Examples of other possible system suitability criteria include background, positive controls, max/min, max/background, slope, IC_{50} (or EC_{50}), and variation around the fitted model.
5. Assess sample suitability for each Test sample. This is done to confirm that the data for each Test sample satisfy necessary assumptions. If a Test sample fails sample suitability, results for that sample are reported as "Fails Sample Suitability." Relative potencies for other Test samples in the assay may still be reported. Most prominent of sample suitability criteria is similarity, whether parallelism for parallel models or equivalence of intercepts for slope-ratio models. For nonlinear models, similarity assessment involves all curve parameters other than EC_{50} (or IC_{50}).
6. For those Test samples in the assay that meet the criterion for similarity to the Standard (i.e., sufficiently similar concentration–response curves or similar straight-line subsets of concentrations), calculate relative potency estimates assuming similarity between Test and Standard, i.e., by analyzing the Test and Standard data together using a model constrained to have exactly parallel lines or curves, or equal intercepts.
7. A single assay is often not sufficient to achieve a reportable value, and potency results from multiple assays can be combined into a single potency estimate. Repeat steps 1–6 multiple times, as specified in the assay protocol or monograph, before determining a final estimate of potency and a confidence interval.
8. Construct a variance estimate and a measure of uncertainty of the potency estimate (e.g., confidence interval). See section 4 *Confidence Intervals*.

A step not shown concerns replacement of missing data. Most modern statistical methodology and software do not require equal numbers at each combination of concentration and sample. Thus, unless otherwise directed by a specific monograph, analysts generally do not need to replace missing values.

3. ANALYSIS MODELS

A number of mathematical functions can be successfully used to describe a concentration–response relationship. The first consideration in choosing a model is the form of the assay response. Is it a number, a count, or a category such as Dead/Alive? The form will identify the possible models that can be considered.

Other considerations in choosing a model include the need to incorporate design elements in the model and the possible benefits of means models compared to regression models. For purposes of presenting the essentials of the model choices, section 3 *Analysis Models* assumes a completely randomized design so that there are no design elements to consider and presents the models in their regression form.

3.1 Quantitative and Qualitative Assay Responses

The terms *quantitative* and *qualitative* refer to the nature of the response of the assay used in constructing the concentration–response model. Assays with either quantitative or qualitative responses can be used to quantify product potency. Note that the *responses* of the assay at the concentrations measured are not the relative potency of the bioassay. Analysts should understand the differences among responses, concentration–response functions, and relative potency.

A quantitative response results in a number on a continuous scale. Common examples include spectrophotometric and luminescence responses, body weights and measurements, and data calculated relative to a standard curve (e.g., cytokine concentration). Models for quantitative responses can be linear or nonlinear (see sections 3.2–3.5).

A qualitative measurement results in a categorical response. For bioassay, qualitative responses are most often quantal, meaning they entail two possible categories such as Positive/Negative, 0/1, or Dead/Alive. Quantal responses may be reported as proportions (e.g., the proportion of animals in a group displaying a property). Quantal models are presented in section 3.6. Qualitative responses can have more than two possible categories, such as end-point titer assays. Models for more than two categories are not considered in this general chapter.

Assay responses can also be counts, such as number of plaques or colonies. Count responses are sometimes treated as quantitative, sometimes as qualitative, and sometimes models specific to integers are used. The choice is often based on the range of counts. If the count is mostly 0 and rarely greater than 1, the assay may be analyzed as quantal and the response is Any/None. If the counts are large and cover a wide range, such as 500 to 2500, then the assay may be analyzed as quantitative, possibly after transformation of the counts. A square root transformation of the count is often helpful in such analyses to better satisfy homogeneity of variances. If the range of counts includes or is near 0 but 0 is not the preponderant value, it may be preferable to use a model specific for integer responses. Poisson regression and negative binomial regression models are often good options. Models specific to integers will not be discussed further in this general chapter.

Assays with quantitative responses may be converted to quantal responses. For example, what may matter is whether some defined threshold is exceeded. The model could then be quantal—threshold exceeded or not. In general, assay systems have more precise estimates of potency if the model uses all the information in the response. Using above or below a threshold, rather than the measured quantitative responses, is likely to degrade the performance of an assay.

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3.2 Overview of Models for Quantitative Responses

In quantitative assays, the measurement is a number on a continuous scale. Optical density values from plate-based assays are such measurements. Models for quantitative assays can be linear or nonlinear. Although the two display an apparent difference in levels of complexity, parallel-line (linear) and parallel-curve (nonlinear) models share many commonalities. Because of the different form of the equations, slope-ratio assays are considered separately (section 3.5 *Slope-Ratio Concentration–Response Models*).

ASSUMPTIONS—The basic parallel-line, parallel-curve, and slope-ratio models share some assumptions. All include a residual term, e, that represents error (variability) which is assumed to be independent from measurement to measurement and to have constant variance from concentration to concentration and sample to sample. Often the residual term is assumed to have a normal distribution as well. The assumptions of independence and equal variances are commonly violated, so the goal in analysis is to incorporate the lack of independence and the unequal variances into the statistical model or the method of estimation.

Lack of independence often arises because of the design or conduct of the assay. For example, if the assay consists of responses from multiple plates, observations from the same plate are likely to share some common influence that is not shared with observations from other plates. This is an example of intraplate correlation. A simple approach for dealing with this lack of independence is to include a block term in the statistical model for plate. With three or more plates this should be a random effects term so that we obtain an estimate of plate-to-plate variability.

In general, the model needs to closely reflect the design. The basic model equations given in sections 3.3–3.5 apply only to completely randomized designs. Any other design will mean additional terms in the statistical model. For example, if plates or portions of plates are used as blocks, one will need terms for blocks.

CALCULATION OF POTENCY—A primary assumption underlying methods used for the calculation of relative potency is that of similarity. Two preparations are similar if they contain the same effective constituent or same effective constituents in the same proportions. If this condition holds, the Test preparation behaves as a dilution (or concentration) of the Standard preparation. Similarity can be represented mathematically as follows. Let F_T be the concentration–response function for the Test, and let F_S be the concentration–response function for the Standard. The underlying mathematical model for similarity is:

$$F_T(z) = F_S(\rho\, z), \quad [3.1]$$

where z represents the concentration and ρ represents the relative potency of the Test sample relative to the Standard sample.

Methods for estimating ρ in some common concentration–response models are discussed below. For linear models, the distinction between parallel-line models (section 3.3 *Parallel-Line Models for Quantitative Response*) and slope-ratio models (section 3.5 *Slope-Ratio Concentration–Response Models*) is based on whether a straight-line fit to log concentration or concentration yields better agreement between the model and the data over the range of concentrations of interest.

3.3 Parallel-Line Models for Quantitative Responses

In this section, a linear model refers to a concentration–response relationship, which is a straight-line (linear) function between the logarithm of concentration, x, and the response, y. y may be the response in the scale as measured or a transformation of the response. The functional form of this relationship is $y = a + bx$. Straight-line fits may be used for portions of nonlinear concentration–response curves, although doing so requires a method for selecting the concentrations to use for each of the Standard and Test samples (see ⟨1032⟩).

MEANS MODEL VERSUS REGRESSION—A linear concentration–response model is most often analyzed with least squares regression. Such an analysis results in estimates of the unknown coefficients (intercepts and slope) and their standard errors, as well as measures of the goodness of fit [e.g., R^2 and root-mean-square error (RMSE)].

Linear regression works best where all concentrations can be used and there is negligible curvature in the concentration–response data. Another statistical method for analyzing linear concentration–response curves is the *means model*. This is an analysis of variance (ANOVA) method that offers some advantages, particularly when one or more concentrations from one or more samples are not used to estimate potency. Because a means model includes a separate mean for each unique combination of sample and dose (as well as block or other effects associated with the design structure) it is equivalent to a saturated polynomial regression model. Hence, a means model provides an estimate of error that is independent of regression lack of fit. In contrast, a regression residual based estimate of error is a mixture of the assay error, as estimated by the means model, combined with lack of fit of the regression model. At least in this sense, the means model error is a better estimate of the residual error variation in an assay system.

PARALLEL-LINE CONCENTRATION–RESPONSE MODELS—If the general concentration–response model (3.1 *Quantitative and Qualitative Assay Responses*) can be made linear in $x = \log(z)$, the resulting equation is then:

$$y = \alpha + \beta\log(z) + e = \alpha + \beta x + e,$$

where e is the residual or error term, and the intercept, α, and slope, β, will differ between Test and Standard. With the parallelism (equal slopes) assumption, the model becomes

$$\begin{aligned} y_S &= \alpha + \beta\log(z) + e = \alpha_S + \beta x + e \\ y_T &= \alpha + \beta\log(\rho z) + e = [\alpha + \beta\log(\rho)] + \beta x + e = \alpha_T + \beta x + e, \end{aligned} \quad [3.2]$$

where S denotes Standard, T denotes Test, $\alpha_S = \alpha$ is the y-intercept for the Standard, and $\alpha_T = \alpha + \beta\log(\rho)$ is the y-intercept for the Test (see *Figure 3.1*).

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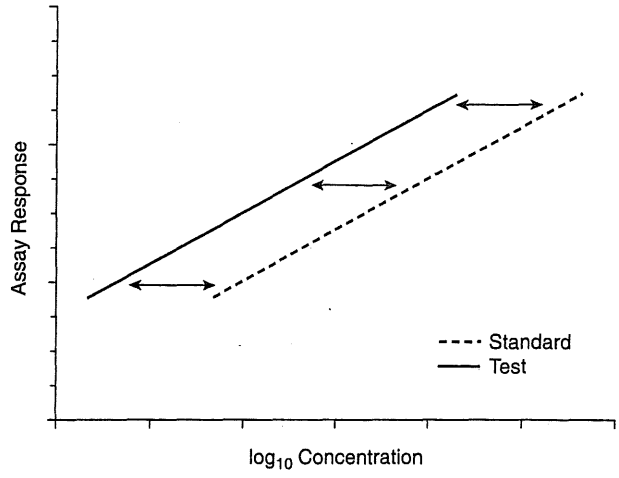

Figure 3.1. Example of parallel-line model.

Where concentration–response lines are parallel, as shown in *Figure 3.1*, a separation or horizontal shift indicates a difference in the level of biological activity being assayed. This horizontal difference is numerically log(ρ), the logarithm of the relative potency, and is found as the vertical distance between the lines α_T and α_S divided by the slope, β. The relative potency is then

$$\rho = \text{antilog}\left(\frac{\alpha_T - \alpha_S}{\beta}\right)$$

ESTIMATION OF PARALLEL-LINE MODELS—Parallel-line models are fit by the method of least squares. If the equal variance assumption holds, the parameters of equation [3.2] are chosen to minimize

$$\sum\left(y - \hat{\alpha}_S - \hat{\delta}T - \hat{\beta}x\right)^2 \qquad [3.3]$$

where the carets denote estimates. This is a linear regression with two independent variables, T and x, where T is a variable that equals 1 for observations from the Test and 0 for observations from the Standard. The summation in equation [3.3] is over all observations of the Test and Standard. If the equal variance assumption does not hold but the variance is known to be inversely proportional to a value, w, that does not depend on the current responses, the y's, and can be determined for each observation, then the method is weighted least squares

$$\sum w\left(y - \hat{\alpha}_S - \hat{\delta}T - \hat{\beta}x\right)^2 \qquad [3.4]$$

Equation 3.4 is appropriate only if the weights are determined without using the response, the y's, from the current data (see ⟨1032⟩ for guidance in determining weights). In equations [3.3] and [3.4] β is the same as the β in equation [3.2] and $\delta = \alpha_T - \alpha_S = \beta \log \rho$. So, the estimate of the relative potency, ρ, is

$$\hat{\rho} = \text{antilog}\left(\hat{\delta}/\hat{\beta}\right)$$

Commonly available statistical software and spreadsheets provide routines for least squares. Not all software can provide weighted analyses.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\delta}/\hat{\beta}$.

MEASUREMENT OF NONPARALLELISM—Parallelism for linear models is assessed by considering the difference or ratio of the two slopes. For the difference, this can be done by fitting the regression model,

$$y = \alpha_S + \delta T + \beta_S x + \gamma x T + e$$

where $\delta = \alpha_T - \alpha_S$, $\gamma = \beta_T - \beta_S$, and T = 1 for Test data and T = 0 for Standard data. Then use the standard t-distribution confidence interval for γ. For the ratio of slopes, fit

$$y = \alpha_S + \delta T + \beta_S x(1 - T) + \beta_T x T + e$$

and use Fieller's Theorem, equation [4.3], to obtain a confidence interval for β_T/β_S.

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3.4 Nonlinear Models for Quantitative Responses

Nonlinear concentration–response models are typically S-shaped functions. They occur when the range of concentrations is wide enough so that responses are constrained by upper and lower asymptotes. The most common of these models is the four-parameter logistic function as given below.

Let y denote the observed response and z the concentration. One form of the four-parameter logistic model is

$$y = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e \qquad [3.5]$$

One alternative, but equivalent, form is

$$y = a_0 + \frac{d}{1 + \text{antilog}\left[M(\log z - b)\right]} + e$$

The two forms correspond as follows:

Lower asymptote: $D = a_0$

Upper asymptote: $A = a_0 + d$

Steepness: $B = M$ (related to the slope of the curve at the EC_{50})

Effective concentration 50% (EC_{50}): $C = \text{antilog}(b)$ (may also be termed ED_{50}).

Any convenient base for logarithms is suitable; it is often convenient to work in log base 2, particularly when concentrations are twofold apart.

The four-parameter logistic curve is symmetric around the EC_{50} when plotted against log concentration because the rates of approach to the upper and lower asymptotes are the same (see *Figure 3.2*). For assays where this symmetry does not hold, asymmetrical model functions may be applied. These models are not considered further in this general chapter.

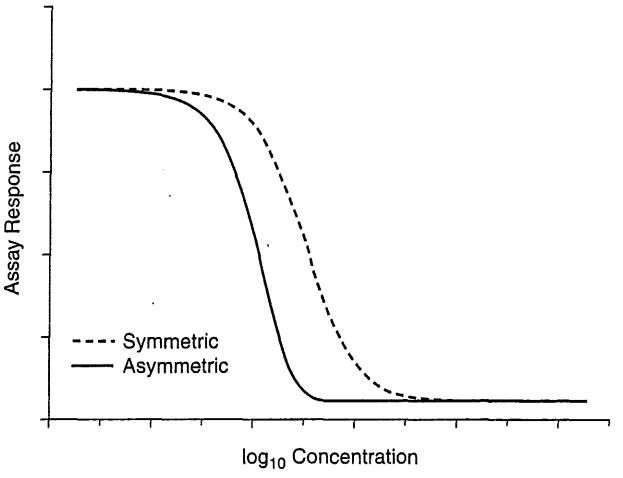

Figure 3.2. Examples of symmetric (four-parameter logistic) and asymmetric sigmoids.

In many assays the analyst has a number of strategic choices to make during assay development (see *Development and Design of Biological Assays* ⟨1032⟩). For example, the responses could be modeled using a transformed response to a four-parameter logistic curve, or the responses could be weighted and fit to an asymmetric sigmoid curve. Also, it is often important to include terms in the model (often random effects) to address variation in the responses (or parameters of the response) associated with blocks or experimental units in the design of the assay. For simple assays where observations are independent, these strategic choices are fairly straightforward. For assays performed with grouped dilutions (as with multichannel pipets), assays with serial dilutions, or assay designs that include blocks (as with multiple plates per assay), it is usually a serious violation of the statistical assumptions to ignore the design structure. For such assays, a good approach involves a transformation that approximates a solution to non-constant variance, non-normality, and asymmetry combined with a model that captures the important parts of the design structure.

PARALLEL-CURVE CONCENTRATION–RESPONSE MODELS—The concept of parallelism is not restricted to linear models. For nonlinear curves, parallel or similar means the concentration–response curves can be superimposed following a horizontal displacement of one of the curves, as shown in *Figure 3.3* for four-parameter logistic curves. In terms of the parameters of equation [3.5], this means the values of A, D, and B for the Test are the same as for the Standard.

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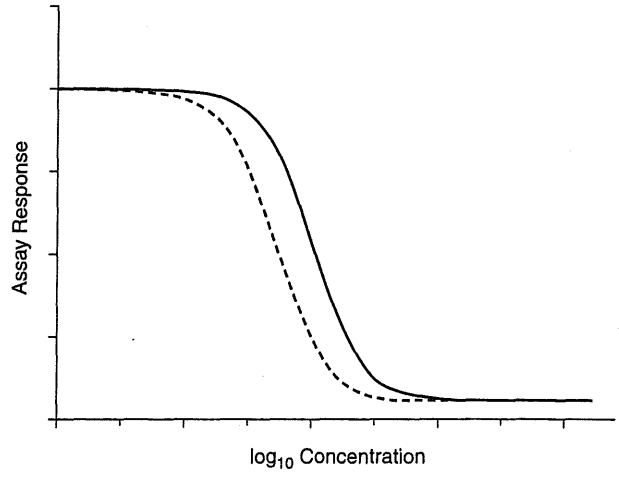

Figure 3.3. Example of parallel curves from a nonlinear model.

The equations corresponding to the figure (with error term, e, added) are

$$y_S = D + \frac{A-D}{1+\left(\frac{z}{C}\right)^B} + e$$

$$y_T = D + \frac{A-D}{1+\left(\frac{\rho z}{C}\right)^B} + e$$

or

$$y_S = D + \frac{A-D}{1+\text{antilog}\left[M(\log z - b)\right]} + e$$

$$y_T = D + \frac{A-D}{1+\text{antilog}\left[M(\log z - b + \log\rho)\right]} + e$$

Log ρ is the log of the relative potency and the horizontal distance between the two curves, just as for the parallel-line model. Because the EC_{50} of the standard is antilog(b) and that of the Test is antilog(b – log ρ) = antilog(b)/ρ, the relative potency is the ratio of EC_{50}'s (standard over Test) when the parallel-curve model holds.

ESTIMATION OF PARALLEL-CURVE MODELS—Estimation of nonlinear, parallel-curve models is similar to that for parallel-line models, possibly after transformation of the response and possibly with weighting. For the four-parameter logistic model, the parameter estimates are found by minimizing:

$$\sum\left(y - \hat{D} - \frac{\hat{A}-\hat{D}}{1+\text{antilog}\left[\hat{M}(\log z - \hat{b} + \hat{r}T)\right]}\right)^2$$

without weighting, or

$$\sum w\left(y - \hat{D} - \frac{\hat{A}-\hat{D}}{1+\text{antilog}\left[\hat{M}(\log z - \hat{b} + \hat{r}T)\right]}\right)^2 \qquad [3.6]$$

with weighting. (As for equation [3.4], equation [3.6] is appropriate only if the weights are determined without using the responses, y's, from the current data.) In either case, the estimate of r is the estimate of the log of the relative potency. For some software, it may be easier to work with d = A – D.

The parameters of the four-parameter logistic function and those of the asymmetric sigmoid models cannot be found with ordinary (linear) least squares regression routines. Computer programs with nonlinear estimation techniques must be used.

Analysts should not use the nonlinear regression fit to assess parallelism or estimate potency if any of the following are present: a) inadequate asymptote information is available; or b) a comparison of pooled error(s) from nonlinear regression to pooled error(s) from a means model shows that the nonlinear model does not fit well; or c) other appropriate measures of goodness of fit show that the nonlinear model is not appropriate (e.g., residual plots show evidence of a "hook").

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, advanced techniques, such as likelihood profiles or bootstrapping are needed to obtain a confidence interval for the log relative potency, r.

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MEASUREMENT OF NONPARALLELISM—Assessment of parallelism for a four-parameter logistic model means assessing the slope parameter and the two asymptotes. During development (see ⟨1032⟩), a decision should be made regarding which parameters are important and how to measure nonparallelism. As discussed in ⟨1032⟩, the measure of nonsimilarity may be a composite measure that considers all parameters together in a single measure, such as the parallelism sum of squares (see ⟨1032⟩), or may consider each parameter separately. In the latter case, the measure may be functions of the parameters, such as an asymptote divided by the difference of asymptotes or the ratio of the asymptotes. For each parameter (or function of parameters), confidence intervals can be computed by bootstrap or likelihood profile methods. These methods are not presented in this general chapter.

3.5 Slope-Ratio Concentration–Response Models

If a straight-line regression fits the nontransformed concentration–response data well, a slope-ratio model may be used. The equations for the slope-ratio model assuming similarity are then:

$$y_S = \alpha + \beta z + e = \alpha + \beta_S z + e$$
$$y_T = \alpha + \beta(\rho z) + e = \alpha + \beta_S \rho z + e = \alpha + \beta_T z + e \qquad [3.7]$$

An identifying characteristic of a slope-ratio concentration–response model that can be seen in the results of a ranging study is that the lines for different potencies from a ranging study have the same intercept and different slopes. Thus, a graph of the ranging study resembles a fan. *Figure 3.4* shows an example of a slope-ratio concentration–response model. Note that the common intercept need not be at the origin.

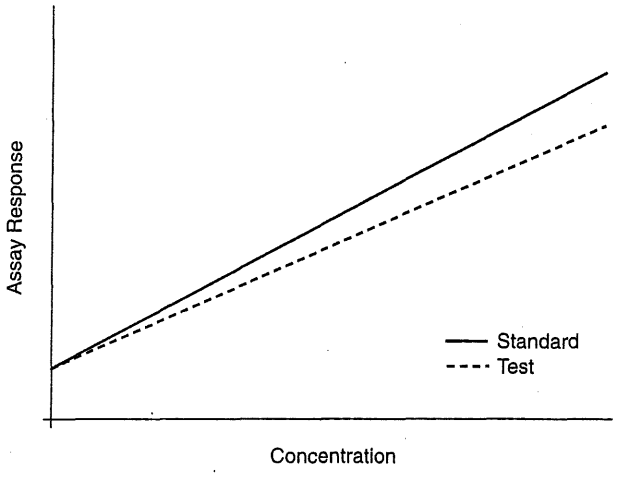

Figure 3.4. Example of slope-ratio model.

An assay with a slope-ratio concentration–response model for measuring relative potency consists, at a minimum, of one Standard sample and one Test sample, each measured at one or more concentrations and, usually, a measured response with no sample (zero concentration). Because the concentrations are not log transformed, they are typically equally spaced on the original, rather than log, scale. The model consists of one common intercept, a slope for the Test sample results, and a slope for the Standard sample results as in equation [3.7]. The relative potency is then found from the ratio of the slopes:

$$\text{Relative Potency} = \text{Test sample slope/Standard sample slope} = \beta\rho/\beta = \rho$$

ASSUMPTIONS FOR AND ESTIMATION OF SLOPE-RATIO MODELS—The assumptions for the slope-ratio model are the same as for parallel-line models: The residual terms are independent, have constant variance, and may need to have a normal distribution. The method of estimation is also least squares. This may be implemented either with or without weighting, as demonstrated in equations [3.8] and [3.9], respectively.

$$\sum\left(y - \hat{\alpha} - \hat{\beta}_S z(1-T) - \hat{\beta}_T zT\right)^2 \qquad [3.8]$$

$$\sum w\left(y - \hat{\alpha} - \hat{\beta}_S z(1-T) - \hat{\beta}_T zT\right)^2 \qquad [3.9]$$

Equation [3.9] is appropriate only if the weights are determined without using the response, the y's, from the current data. This is a linear regression with two independent variables, $z(1 - T)$ and zT, where $T = 1$ for Test data and $T = 0$ for Standard data. $\hat{\beta}_T$ is the estimated slope for the Test, $\hat{\beta}_S$ the estimated slope for the Standard, and then the estimate of relative potency is

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$$R = \hat{\beta}_T / \hat{\beta}_S$$

Because the slope-ratio model is a linear regression model, most statistical packages and spreadsheets can be used to obtain the relative potency estimate. In some assay systems, it is sometimes appropriate to omit the zero concentration (e.g., if the no-dose controls are handled differently in the assay) and at times one or more of the high concentrations (e.g., if there is a hook effect where the highest concentrations do not have the highest responses). The discussion about using a means model and selecting subsets of concentrations for straight parallel-line bioassays applies to slope-ratio assays as well.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to

$$\hat{\beta}_T / \hat{\beta}_S$$

MEASUREMENT OF NONSIMILARITY—For slope-ratio models, statistical similarity corresponds to equal intercepts for the Standard and Test. To assess the similarity assumption it is necessary to have at least two nonzero concentrations for each sample. If the intercepts are not equal, equation [3.7] becomes

$$y_S = \alpha_S + \beta_S z + e$$

$$y_T = \alpha_T + \beta_T z + e$$

Departure from similarity is typically measured by the difference of intercepts, $\alpha_T - \alpha_S$. An easy way to obtain a confidence interval is to fit the model,

$$y = \alpha_S + \delta T + \beta_S z(1 - T) + \beta_T z T + e,$$

where $\delta = \alpha T - \alpha_S$ and use the standard t-distribution-based confidence interval for δ.

3.6 Dichotomous (Quantal) Assays

For quantal assays the assay measurement has a dichotomous or binary outcome, e.g., in animal assays the animal is dead or alive or a certain physiologic response is or is not observed. For cellular assays, the quantal response may be whether there is or is not a response beyond some threshold in the cell. In cell-based viral titer or colony-forming assays, the quantal response may be a limit of integer response such as an integer number of particles or colonies. When one can readily determine if any particles are present—but not their actual number—then the assay can be analyzed as quantal. Note that if the reaction can be quantitated on a continuous scale, as with an optical density, then the assay is not quantal.

MODELS FOR QUANTAL ANALYSES—The key to models for quantal responses is to work with the probability of a response (e.g., probability of death), in contrast to quantitative responses for which the model is for the response itself. For each concentration, z, a treated animal, as an example, has a probability of responding to that concentration, P(z). Often the curve P(z) can be approximated by a sigmoid when plotted against the logarithm of concentration, as shown in *Figure 3.5*. This curve shows that the probability of responding increases with concentration. The concentration that corresponds to a probability of 0.5 is the EC_{50}.

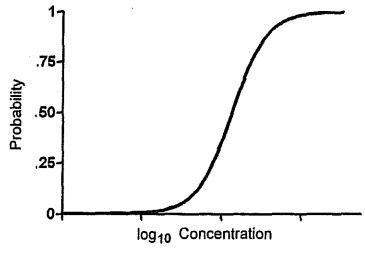

Figure 3.5. Example of sigmoid for P(z).

The sigmoid curve is usually modeled based on the normal or logistic distribution. If the normal distribution is used, the resulting analysis is termed probit analysis, and if the logistic is used the analysis is termed logit or logistic analysis. The probit and logit models are practically indistinguishable, and either is an acceptable choice. The choice may be based on the availability of software that meets the laboratory's analysis and reporting needs. Because software is more commonly available for logistic models (often under the term logistic regression) this discussion will focus on the use and interpretation of logit analysis. The considerations discussed in this section for logit analysis (using a logit transformation) apply as well to probit analysis (using a probit transformation).

LOGIT MODEL—The logit model for the probability of response, P(z), can be expressed in two equivalent forms. For the sigmoid,

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$$P(z) = \frac{1}{1 + \text{antilog}\,[-\beta_0 - \beta_1 \log(z)]} = \frac{1}{1 + (z/ED_{50})^{-\beta_1}}$$

where $\log(ED_{50}) = -\beta_0/\beta_1$. An alternative form shows the relationship to linear models:

$$\text{logit transform of P} = \log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) \qquad [3.10]$$

The linear form is usually shown using natural logs and is a useful reminder that many of the considerations, in particular linearity and parallelism, discussed for parallel-line models in section 3.3 *Parallel-Line Models for Quantitative Responses* apply to quantal models as well.

For a logit analysis with Standard and Test preparations, let T be a variable that takes the value 1 for animals receiving the Test preparation and 0 for animals receiving the Standard. Assuming parallelism of the Test and Standard curves, the logit model for estimating relative potency is then:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T$$

The log of the relative potency of the Test compared to the Standard preparation is then β_2/β_1. The two curves in *Figure 3.6* show parallel Standard and Test sigmoids. (If the corresponding linear forms equation [3.10] were shown, they would be two parallel straight lines.) The log of the relative potency is the horizontal distance between the two curves, in the same way as for the linear and four-parameter logistic models given for quantitative responses (sections 3.3 *Parallel-Line Models for Quantitative Responses* and 3.4 *Nonlinear Models for Quantitative Responses*).

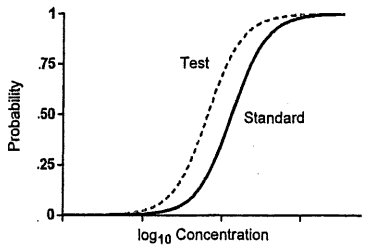

Figure 3.6. Example of Parallel Sigmoid Curves.

ESTIMATING THE MODEL PARAMETERS AND RELATIVE POTENCY—Two methods are available for estimating the parameters of logit and probit models: maximum likelihood and weighted least squares. The difference is not practically important, and the laboratory can accept the choice made by its software. The following assumes a general logistic regression software program. Specialized software should be similar.

Considering the form of equation [3.10], one observes a resemblance to linear regression. There are two independent variables, x = log(z) and T. For each animal, there is a yes/no dependent variable, often coded as 1 for yes or *response* and 0 for *no* or *no response*. Although bioassays are often designed with equal numbers of animals per concentration, that is not a requirement of analysis. Utilizing the parameters estimated by software, which include β_0, β_1, and β_2 and their standard errors, one obtains the estimate of the natural log of the relative potency:

$$\text{Estimate of log of relative potency} = \frac{\hat{\beta}_2}{\hat{\beta}_1}$$

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\beta}_2/\hat{\beta}_1$. The confidence interval for the relative potency is then [antilog(L), antilog(U)], where [L, U] is the confidence interval for the log relative potency.

ASSUMPTIONS—Assumptions for quantal models have two parts. The first concerns underlying assumptions related to the probability of response of each animal or unit in the bioassay. These are difficult to verify assumptions that depend on the design of the assay. The second part concerns assumptions for the statistical model for P(z). Most important of these are parallelism and linearity. These assumptions can be checked much as for parallel-line analyses for quantitative responses.

In most cases, quantal analyses assume a standard binomial probability model, a common choice of distribution for dichotomous data. The key assumptions of the binomial are that at a given concentration each animal treated at that concentration has the same probability of responding and the results for any animal are independent from those of all other animals. This basic set of assumptions can be violated in many ways. Foremost among them is the presence of litter effects, where animals from the same litter tend to respond more alike than do animals from different litters. Cage effects, in which the environmental conditions or care rendered to any specific cage makes the animals from that cage more or less likely to respond to experimental treatment, violates the equal-probability and independence assumptions. These assumption violations and

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others like them (that could be a deliberate design choice) do not preclude the use of logit or probit models. Still, they are indications that a more complex approach to analysis than that presented here may be required (see ⟨1032⟩).

CHECKING ASSUMPTIONS—The statistical model for P(z) assumes linearity and parallelism. To assess parallelism, equation [3.10] may be modified as follows:

$$\log\left(\frac{P}{1-P}\right)=\beta_0+\beta_1\log(z)+\beta_2 T+\beta_3 T*\log(z)$$

Here, β_3 is the difference of slopes between Test and Standard and should be sufficiently small. [The T*log(z) term is known as an *interaction term* in statistical terminology.] The measure of nonparallelism may also be expressed in terms of the ratio of slopes, $(\beta_1+\beta_3)/\beta_1$. For model-based confidence intervals for these measures of nonparallelism, bootstrap or profile likelihood methods are recommended. These methods are not covered in this general chapter.

To assess linearity, it is good practice to start with a graphical examination. In accordance with equation [3.10], this would be a plot of log[(y + 0.5)/(n – y + 0.5)] against log(concentration), where y is the total number of responses at the concentration and n is the number of animals at that concentration. (The 0.5 corrections improve the properties of this calculation as an estimate of log[P/(1 – P)].) The lines for Test and Standard should be parallel straight lines as for the linear model in quantitative assays. If the relationship is monotonic but does not appear to be linear, then the model in [3.10] can be extended with other terms. For example, a quadratic term in log(concentration) could be added: $[\log(\text{concentration})]^2$. If concentration needs to be transformed to something other than log concentration, then the quantal model analogue of slope-ratio assays is an option. The latter is possible but sufficiently unusual that it will not be discussed further in this general chapter.

OUTLIERS—Assessment of outliers is more difficult for quantal assays than for quantitative assays. Because the assay response can be only *yes* or *no*, no individual response can be unusual. What may appear to fall into the outlier category is a single response at a low concentration or a single no-response at a high concentration. Assuming that there has been no cause found (e.g., failure to properly administer the drug to the animal), there is no statistical basis for distinguishing an outlier from a rare event.

ALTERNATIVE METHODS—Alternatives to the simple quantal analyses outlined here may be acceptable, depending on the nature of the analytical challenge. One such challenge is a lack of independence among experimental units, as may be seen in litter effects in animal assays. Some of the possible approaches that may be employed are Generalized Estimating Equations (GEE), generalized linear models, and generalized linear mixed-effects models. A GEE analysis will yield standard errors and confidence intervals whose validity does not depend on the satisfaction of the independence assumption.

There are also methods that make no particular choice of the model equation for the sigmoid. A commonly seen example is the Spearman–Kärber method.

4. CONFIDENCE INTERVALS

A report of an assay result should include a measure of the uncertainty of that result. This is often a standard error or a confidence interval. An interval (c, d), where c is the lower confidence limit and d is the upper confidence limit, is a 95% confidence interval for a parameter (e.g., relative potency) if 95% of such intervals upon repetition of the experiment would include the actual value of the parameter. A confidence interval may be interpreted as indicating values of the parameter that are consistent with the data. This interpretation of a confidence interval requires that various assumptions be satisfied. Assumptions also need to be satisfied when the width or half width [(d-c)/2] is used in a monograph as a measure of whether there is adequate precision to report a potency. The interval width is sometimes used as a suitability criterion without the confidence interpretation. In such cases the assumptions need not be satisfied.

Confidence intervals can either be *model-based* or *sample-based*. A model-based interval is based on the standard errors for each of the one or more estimates of log relative potency that come from the analysis of a particular statistical model. Model-based intervals should be avoided if sample-based intervals are possible. Model-based intervals require that the statistical model correctly incorporate all the effects and correlations that influence the model's estimate of precision. These include but are not be limited to serial dilution and plate effects. Section 4.3 *Model-Based Methods* describes Fieller's Theorem, a commonly used model-based interval.

Sample-based methods combine independent estimates of log relative potency. Multiple assays may arise because this was determined to be required during development and validation or because the assay procedure fixes a maximum acceptable width of the confidence interval and two or more independent assays may be needed to meet the specified width requirement. Some sample-based methods do not require that the statistical model correctly incorporate all effects and correlations. However, this should not be interpreted as dismissing the value of addressing correlations and other factors that influence within-assay precision. The within-assay precision is used in similarity assessment and is a portion of the variability that is the basis for the sample-based intervals. Thus minimizing within-assay variability to the extent practical is important. Sample-based intervals are covered in section 4.2 *Combining Independent Assays (Sample-Based Confidence Interval Methods)*.

4.1 Combining Results from Multiple Assays

In order to mitigate the effects of variability, it is appropriate to replicate independent bioassays and combine their results to obtain a single reportable value. That single reportable value (and not the individual assay results) is then compared to any applicable acceptance criteria. During assay development and validation, analysts should evaluate whether it is useful to combine the results of such assays and, if so, in what way to proceed.

There are two primary questions to address when considering how to combine results from multiple assays:

Are the assays mutually independent?

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A set of assays may be regarded as mutually independent when the responses of one do not in any way depend on the distribution of responses of any of the others. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined or the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other assays. Assays on successive days using the original and retained dilutions of the Standard, therefore, are not independent assays. Similarly, if the responses, particularly the potency, depend on other reagents that are shared by assays (e.g., cell preparations), the assays may not be independent.

Assays need not be independent in order for analysts to combine results. However, methods for independent assays are much simpler. Also, combining dependent assay results may require assumptions about the form of the correlation between assay results that may be, at best, difficult to verify. Statistical methods are available for dependent assays, but they are not presented in this general chapter.

Are the results of the assays homogeneous?

Homogeneous results differ only because of random within-assay errors. Any contribution from factors associated with intermediate precision precludes homogeneity of results. Intermediate precision factors are those that vary between assays within a laboratory and can include analyst, equipment, and environmental conditions. There are statistical tests for heterogeneity, but lack of statistically significant heterogeneity is not properly taken as assurance of homogeneity and so no test is recommended. If analysts use a method that assumes homogeneity, homogeneity should be assessed during development, documented during validation, and monitored during ongoing use of the assay.

Additionally, before results from assays can be combined, analysts should consider the scale on which that combination is to be made. In general, the combination should be done on the scale for which the parameter estimates are approximately normally distributed. Thus, for relative potencies based on a parallel-line, parallel-curve, or quantal method, the relative potencies are combined in the logarithm scale.

4.2 Combining Independent Assays (Sample-Based Confidence Interval Methods)

Analysts can use several methods for combining the results of independent assays. A simple method described below (Method 1) assumes a common distribution of relative potencies across the assays and is recommended. A second procedure is provided and may be useful if homogeneity of relative potency across assays can be documented. A third alternative is useful if the assumptions for Methods 1 and 2 are not satisfied. Another alternative, analyzing all assays together using a linear or nonlinear mixed-effects model, is not discussed in this general chapter.

METHOD 1—INDEPENDENT ASSAY RESULTS FROM A COMMON ASSAY DISTRIBUTION—The following is a simple method that assumes independence of assays. It is assumed that the individual assay results (logarithms of relative potencies) are from a common normal distribution with some nonzero variance. This common distribution assumption requires that all assays to be combined used the same design and laboratory procedures. Implicit is that the relative potencies may differ between the assays. This method thus captures interassay variability in relative potency. Note that the individual relative potencies should not be rounded before combining results.

Let R_i denote the logarithm of the relative potency of the ith assay of N assay results to be combined. To combine the N results, the mean, standard deviation, and standard error of the R_i are calculated in the usual way:

$$\text{Mean } \bar{R} = \sum_{i=1}^{N} R_i/N$$

$$\text{Standard Deviation } S = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(R_i - \bar{R})^2}$$

$$\text{Standard Error } SE = S/\sqrt{N}$$

A 100(1 – α)% confidence interval is then found as

$$\bar{R} \pm t_{N-1,\alpha/2}SE,$$

where $t_{N-1,\alpha/2}$ is the upper α/2 percentage point of a t-distribution with N – 1 degrees of freedom. The quantity $t_{N-1,\alpha/2}SE$ is the expanded uncertainty of $\bar{R}$. The number, N, of assays to be combined is usually small, and hence the value of t is usually large.

Because the results are combined in the logarithm scale, the combined result can be reported in the untransformed scale as a confidence interval for the geometric mean potency, estimated by antilog($\bar{R}$),

$$\text{antilog}\left(\bar{R} - t_{N-1,\alpha/2}SE\right),\ \text{antilog}\left(\bar{R} \pm t_{N-1,\alpha/2}SE\right)$$

METHOD 2—INDEPENDENT ASSAY RESULTS, HOMOGENEITY ASSUMED—This method can be used provided the following conditions are fulfilled:

1. The individual potency estimates form a homogeneous set with regard to the potency being estimated. Note that this means documenting (usually during development and validation) that there are no contributions to between-assay variability from intermediate precision factors. The individual results should appear to be consistent with homogeneity. In particular, differences between them should be consistent with their standard errors.
2. The potency estimates are derived from independent assays.
3. The number of degrees of freedom of the individual residual errors is not small. This is required so that the weights are well determined.

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When these conditions are not fulfilled, this method cannot be applied and Method 1, Method 3, or some other method should be used. Further note that Method 2 (because it assumes no inter-assay variability) often results in narrower confidence intervals than Method 1, but this is not sufficient justification for using Method 2 absent satisfaction of the conditions listed above.

CALCULATION OF WEIGHTING COEFFICIENTS—It is assumed that the results of each of the N assays have been analyzed to give N estimates of log potency with associated confidence limits. For each assay, i, the logarithmic confidence interval for the log potency or log relative potency and a value L_i are obtained by subtracting the lower confidence limit from the upper. (This formula, using the L_i, accommodates asymmetric confidence intervals such as from Fieller's Theorem, section 4.3 *Model-Based Methods*). A weight W_i for each value of the log relative potency, R_i, is calculated as follows, where t_i has the same value as that used in the calculation of confidence limits in the ith assay:

$$W_i = \frac{4t_i^2}{L_i^2} \qquad [4.1]$$

CALCULATION OF THE WEIGHTED MEAN AND CONFIDENCE LIMITS—The products W_iR_i are formed for each assay, and their sum is divided by the total weight for all assays to give the weighted mean log relative potency and its standard error as follows:

$$\text{Mean } \bar{R} = \sum_{i=1}^{N} W_i R_i \Big/ \sum_{i=1}^{N} W_i$$

$$\text{Standard Error SE} = 1/\sqrt{\sum_{i=1}^{N} W_i}$$

A 100(1 – α)% confidence interval in the log scale is then found as

$$\bar{R} \pm t_{k,\alpha/2}SE \qquad [4.2]$$

where $t_{k,\alpha/2}$ is the upper α/2 percentage point of a t-distribution with degrees of freedom, k, equal to the sum of the number of degrees of freedom for the error mean squares in the individual assays. This confidence interval can then be transformed back to the original scale as for Method 1.

METHOD 3—INDEPENDENT ASSAY RESULTS, COMMON ASSAY DISTRIBUTION NOT ASSUMED—Method 3 is an approximate method that may be considered if the conditions for Method 1 (common assay distribution) or Method 2 (homogeneity) are not met.

The observed variation then has two components:

- the intra-assay variation for assay i:

$$s_i^2 = 1/W_i$$

- the inter-assay variation:

$$s_B^2 = \frac{1}{N-1}\sum_{i=1}^{N}(R_i - \bar{R})^2 - \frac{1}{N}\sum_{i=1}^{N} s_i^2$$

For each assay, a weighting coefficient is then calculated as

$$W_i' = \frac{1}{s_i^2 + s_B^2}$$

which replaces W_i in equation [4.1] and where t in equation [4.2] is often approximated by the value 2.

4.3 Model-Based Methods

Many confidence intervals are of the form:

Confidence interval = value ± k times the standard error of that value.

For such cases, as long as the multiplier k can be easily determined (e.g., from a table of the t-distribution), reporting the standard error and the confidence interval are largely equivalent because the confidence interval is then easily determined from the standard error. However, the logarithms of relative potencies for parallel-line models and some parameterizations of nonlinear models and the relative potencies from slope-ratio models are ratios. In such cases, the confidence intervals are not symmetric around the estimated log relative potency or potency, and Fieller's Theorem is needed. For these asymmetric cases the confidence interval should be reported because the standard error by itself does not capture the asymmetry.

Fieller's Theorem is the formula for the confidence interval for a ratio. Let R = a/b be the ratio for which we need a confidence interval. For the estimates of a and b, we have their respective standard errors, SE_a and SE_b, and a covariance between them, denoted Cov. (The covariance is a measure of the degree to which the estimates of a and b are related and is proportional to

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the correlation between the estimates of a and b.) The covariance may be 0, as for some parameterizations of standard parallel-line analyses, but it need not be. The confidence interval for R then is as follows:

$$(R_L,R_U)=\frac{\left\{\hat{R}-\frac{gCov}{SE_b^2}\pm\frac{t}{\hat{b}}\sqrt{(1-g)SE_a^2+\hat{R}^2SE_b^2-2\hat{R}Cov+\frac{gCov^2}{SE_b^2}}\right\}}{1-g}$$

where

$$g=\frac{t^2SE_b^2}{\hat{b}^2}$$

and t is the appropriate t deviate value that will depend on the sample size and confidence level chosen (usually 95%). If g > 1, it means that the denominator, $\hat{b}$, is not statistically significantly different from 0 and the use of the ratio is not sensible for those data.

For those cases where the estimates of a and b are statistically uncorrelated (Cov = 0), the confidence interval formula simplifies to

$$(R_L,R_U)=\frac{\left\{\hat{R}\pm\frac{t}{\hat{b}}\sqrt{(1-g)SE_a^2+\hat{R}^2SE_b^2}\right\}}{1-g} \qquad [4.3]$$

5. ADDITIONAL SOURCES OF INFORMATION

A variety of statistical methods can be used to analyze bioassay data. This chapter presents several methods, but many other similar methods could also be employed. Additional information and alternative procedures can be found in the references listed below and other sources.

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⟨1039⟩ CHEMOMETRICS

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1. INTRODUCTION

1.1 Scope and Purpose

This chapter provides guidance regarding scientifically sound practices for the chemometric analysis and interpretation of typical multivariate data for compendial and industrial applications. Established chemometric practices, including calibration and validation, for applications using different analytical technologies (e.g., spectroscopic, chromatographic, and others) and for different purposes (e.g., fingerprinting, identification, classification, properties prediction, and others) are discussed under a lifecycle approach. Both qualitative and quantitative applications are described.

The chapter discusses how method quality and performance are ensured through the proper lifecycle management of a chemometrics-based model, including the selection of appropriate algorithms, calibration, validation, verification, transfer, and ongoing maintenance steps.

This chapter may be viewed as a supplement to other guidance chapters such as *Analytical Data—Interpretation and Treatment* ⟨1010⟩, which are mainly concerned with the analysis and interpretation of univariate data.

[NOTE—It should not be inferred that the multivariate analysis tools mentioned in this chapter form an exhaustive list. Other equally valid models may be used at the discretion of the manufacturer and other users of this chapter.]

1.2 Content Summary of Document

The mind map below (*Figure 1*) provides a visual representation of the content of this chapter. This diagram is meant to assist the reader by showing how the various concepts and practices of chemometrics relate to each other.

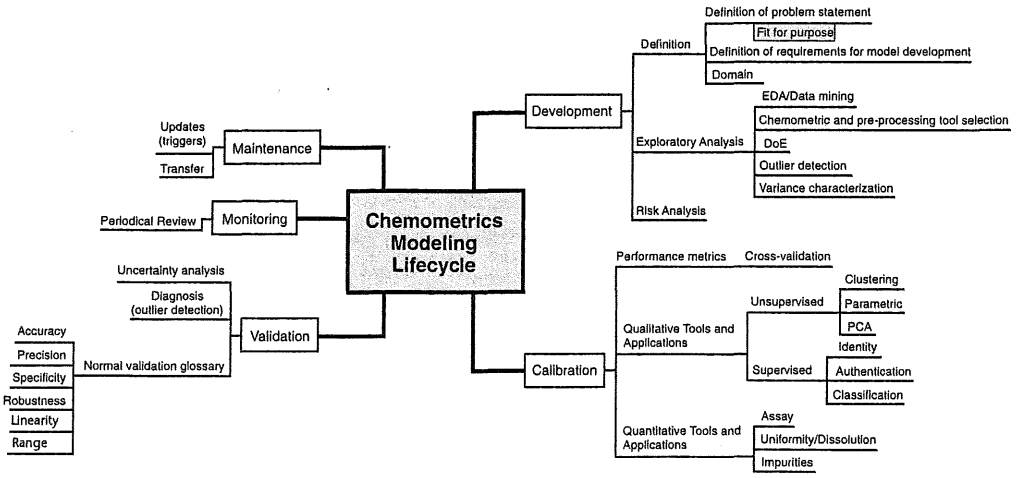

Figure 1. Content summary of document.

1.3 Audience

This chapter provides direction for both the chemometrician applying chemometric techniques to develop a model for a given application and the analyst that runs the model within an analytical procedure. The chemometrician will find support regarding algorithm selection for a given application and guidance for ensuring model performance throughout its lifecycle. The analyst will gain insight regarding the strengths and limitations of the chemometrics techniques as applied to support their applications.

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2. WHAT IS CHEMOMETRICS?

Chemometrics was originally defined as the chemical discipline that uses mathematical, statistical, and other methods that employ formal logic to accomplish two objectives: 1) to design or select optimal measurement procedures and experiments, and 2) to provide the maximum amount of relevant chemical information by analyzing chemical data. (*1*,*2*) More specifically, "chemometrics" has come to mean the application of multivariate methods for the analysis of chemical or related data, although the algorithms in question may be used to extract information out of almost any measured data, regardless of origin—chemical, physical, biological, pharmaceutical, or others. This chapter does not focus on the development of optimal procedures or methods and the applied design of experiments (DoE), but rather on the analysis of multidimensional data collected from an analytical instrument, such as spectroscopic and chromatographic data. A multivariate data set (i.e., multivariate data obtained for a number of samples or objects) forms an $m \times n$ data table or matrix. The matrix is represented by **X**, with m as the number of samples or objects and n as the number of variables measured for each sample (*Figure 2*).

Consequently, the data analysis techniques considered in this chapter will be multivariate in nature. Depending on the purpose of the data treatment, different tools will be applied. Initially, the data handling techniques can be divided into two categories: unsupervised and supervised. The unsupervised tools use only the **X** matrix to extract information, but in supervised data analysis, in addition to the **X** matrix the samples are also described by a **y** vector. This is an $m \times 1$ table containing property information for each sample (e.g., concentration, enzyme inhibition activity). The supervised data analysis techniques are used to build a model between the **X** matrix and the **y** vector. In chemometric modeling, the equations provided are data driven to empirically describe the underlying variance in the data for either unsupervised or supervised purposes. The different techniques or tools applied for the different purposes are discussed in more detail in *4. Applications of Chemometrics*.

The most commonly used unsupervised technique (principal component analysis, or PCA) and supervised technique (partial least squares regression, or PLS) are by nature latent projection approaches (see *Figure 2*), which transform a large number of potentially correlated **X** variables, such as intensities at different retention times or wavelengths, into a possibly smaller number of uncorrelated variables (principal components, or PCs; latent variables). As can be seen in *Figure 2*, the original n-dimensional space was transformed to a two-PC space. When samples from the original data space are projected onto this lower-dimensionality space, the resulting sample coordinates are called scores (**T**). Visualization of the scores along two or more PCs forms a score plot that contains information on the relationships among different samples. The loadings (**P**) are a linear combination of the original variables and the coefficients or weights used in this linear combination. The loadings for specific latent variables also can be plotted in what is called a loading plot. The loading plot contains information on the relative importance among the original variables. Both scores and loading plots enable visualization and understanding of the underlying data structure (i.e., the presence of groups/clusters and/or outliers) within a reduced dimensional space. The variable matrix (**E**) produced by the model is defined as the residual error. The sample information along axes of common variance is captured by the model's PCs. Variance unaccounted for by these PCs (residual error) is left for each sample at each variable, forming the residual matrix (**E**).

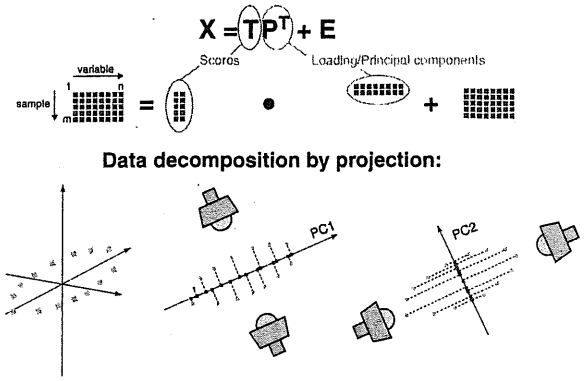

Figure 2. Schematic representation of latent projection techniques.

3. MODEL LIFECYCLE

The development of a chemometric model, as part of an analytical procedure, aims to fulfill a predefined, intended purpose. The intended purpose is typically a statement describing what to measure, the output format, and the level of performance needed with the result, and should be in accordance with the analytical target profile (ATP). The ATP may specify performance criteria for key characteristics of the analytical procedure output and/or decision risk probabilities expected in routine use of the analytical procedure.

Calibration of the model encompasses an iterative process that involves selection of the sample set to be used to develop the chemometric model, tuning of an appropriate chemometric algorithm with the necessary preprocessing algorithm, and evaluation of model performance according to predefined metrics for the ATP. During the validation stage, the method performance is demonstrated to fulfill the intended purpose and ATP. Both the knowledge gained during calibration and the assessment of specific metrics and corresponding limits are used to evaluate performance and define a method maintenance protocol that will be used for the monitoring stage, before deployment to routine use. Changes that may have an impact on model performance should trigger a defined set of activities to update it. Model update may also be triggered by the necessity

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of performing method transfer, in the course of its lifecycle, to different equipment or another site. The extent of the revalidation will be defined by the magnitude of the model update. *Figure 3* shows a schematic representation of the described workflow.

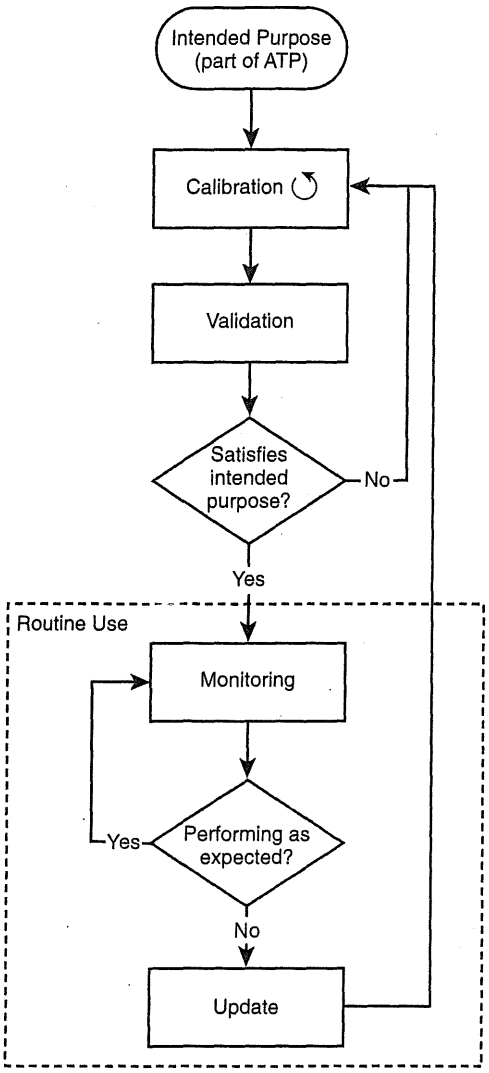

Figure 3. Schematic representation of the lifecycle workflow of a chemometric model.

3.1 Model Development: Calibration

The goal of multivariate calibration is to develop a model that links predictor variables (e.g., absorbance values for the range of spectral wavelengths) with response variables such as concentrations of certain chemical compounds. Response variables are typically more difficult or costly to obtain than predictor variables. Calibration is a critical step because details of the final model are influenced by many factors including the selection of samples, variables, an algorithm, and preprocessing options, as well as the quality of the reference method initially used (where applicable) to measure the response variable(s). Thus, the order of individual components of model calibration presented in this section does not always represent the order of operation, and individual steps might be repeated in an iterative fashion depending upon the outcome of subsequent steps. To perform well, the calibration model should be robust to intended variations.

SAMPLE SELECTION

Selection of samples for the calibration model is a critical step in model development. Scientifically sound rationales must be used to select representative calibration samples with respect to two criteria: the type/range of variability, and the number of samples. The range of response values in selected samples should cover the entire expected range of response variation for the intended application. The range and type of variation in predictors may include relevant variability in factors other than the property being predicted, such as particle size, different lots of ingredients used for sample preparation, analyst-to-analyst and day-to-day variation, and other sources of variation. In the manufacturing setting, batches of samples representing within and outside specification should be included. A risk-based approach is recommended for deciding which factors and variability will

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be included in the calibration samples. Performing a failure mode and effect analysis (FMEA) or a feasibility study to evaluate the effects of such factors is one of the commonly used approaches.

The required number of samples increases as the complexity of the calibration model (i.e., the type and the range of variability in both response and predictor variables) increases. In general, the larger the number of samples, the higher the probability that correct results can be achieved throughout the range of calibration. Meanwhile, the distribution of the calibration samples also deserves attention. A uniform distribution of the samples is preferred, although it is not required in all cases, depending on the specifics of the application. Scientifically sound rationales must be in place to justify the number and the distribution of calibration samples. When obtaining new calibration samples becomes costly, the DoE and/or historical database approaches are commonly used alternatives to build calibration models.

Data obtained from selected samples (i.e., a selected number of rows of the **X** matrix in *Figure 2*) should undergo further triage. Exploratory data analysis (see *Qualitative Application Examples*) may be valuable for understanding data structure, detecting potential clusters or groups of samples, identifying outliers, and selecting representative samples. Outliers may be detected using metrics that describe how well a given observation fits within model space (e.g., Hotelling's T^2 for latent variable methods) and distance to the model subspace (e.g., residual for latent variable methods).

PREPROCESSING

The goal of preprocessing is to deploy mathematical transformations on the column of the **X** matrix (*Figure 2*) to amplify variation of interest within the data set and attenuate extraneous variation for both variables and observations. Preprocessing is an important initial step for most data analyses, and can be applied iteratively with variable selection. The initial selection of preprocessing options should be guided by an understanding of the sample data and/or the underlying analytical technique. Guidance may also be obtained through exploratory multivariate data analyses (see *Qualitative Application Examples*). Initial selections can be modified or refined within the context of subsequent model performance optimization. This process is almost always cyclic in practice; comparison across different preprocessing strategies leads to a better understanding of the data, which can further refine the preprocessing to maximize the signal-to-noise ratio.

However, there are two points of caution. Preprocessing should be used judiciously because: 1) overprocessing can attenuate the signal and inflate undesirable variations; and 2) many chemometric methods can accommodate noisy and visually unappealing data. Furthermore, in some instances the preprocessing has been accomplished on the instrument; many algorithms used by instrument vendors are proprietary, and it may be impossible to know exactly what modifications have been made to the data. The chemometrician must be aware of, and cautious about, any preprocessing already applied in the reported raw data.

Preprocessing may consist of transformation, normalization, and/or other mathematical treatment of data. Preprocessing of samples (rows of data matrix) may include mean or median centering, scaling, and other procedures. Variables (columns of data matrix) can also be transformed, aligned (e.g., time warping), or scaled (e.g., autoscaling). Mean centering is probably the most common preprocessing method. For example, the use of mean-centered data in the regression model removes the intercept term, resulting in a potentially parsimonious model. It is also common to remove the linear baseline (e.g., bias/offset correction) or polynomial interferences or to apply digital filtering techniques such as derivatives, wavelets, Fourier filter, or a Savitzky-Golay filter to remove or attenuate linear offsets and noise contribution. Normalization is the scaling of observations, giving objects similar impact on model creation by: 1) accounting for factors other than the analyte of interest that impact measurement (e.g., sample path length variability caused by particle size, sample volume, or tablet thickness); 2) correcting for variation in concentration or mass of an analyte for qualitative models; and 3) attenuating measurement artifacts (e.g., instrument performance drift). When variables are highly correlated, such as in spectroscopic measurements, normalization is typically performed via standard normal variate (SNV) and multiplicative scatter correction (MSC). These normalizations are typically conducted on each sample (row).

In cases where variables have different units, sensitivities, or signal-to-noise ratios, variable scaling is typically applied to correct for that; autoscaling is typically used, but the analyst may consider other alternatives, such as using the pooled standard deviations of measurement replicates as scaling/weight parameters. These normalizations are typically conducted on each variable (column). No guidelines exist for deciding which preprocessing approach is the best for a given data set. It is often a trial-and-error process as the analyst applies different preprocessing techniques to find the best one. Combinations of preprocessing techniques can be applied to a given data set as well.

ALGORITHM SELECTION

Many different algorithms have been tried with varying degrees of success in chemometrics applications. The methods include relatively simple ones such as multivariate linear regression and its modifications (robust or weighted regression); the widely used latent variables approaches such as principal components regression (PCR) and PLS; local methods such as k-nearest neighbors (kNN); and the more sophisticated methods such as support vector machines (SVM) and artificial neural networks. More details on commonly used algorithmic tools can be found in *4. Applications of Chemometrics*. In general, it is difficult to predict which algorithm will produce the best results for a particular data set. The multitude of choices for sample selection, variable selection, and data normalization and preprocessing—as well as the combination of tuning parameters for each algorithm—could significantly affect the performance of the calibration model. Sometimes, the choice of algorithm even iterates with the steps of sample selection, variable selection, and preprocessing. Thus, the choice of algorithm may depend on the task at hand, software availability, and the subject matter expert's familiarity with the method. Another consideration is that some algorithms provide useful tools for diagnostics and interpretation—such as PLS scores, loadings, and coefficients plots, whereas others are sometimes referred to as "black boxes" because their inner workings can be difficult to interpret (e.g., neural networks).

It is important to keep in mind that many algorithms are empirical in nature. Almost always, some kind of model can be developed. Thus, the results should be evaluated critically to ensure that results generated by the model are relevant and correct,

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and that model performance meets the requirements of the ATP. This can be accomplished by cross-validation, and ultimately, by validation with the independent test data set that was not used to develop the model.

VARIABLE SELECTION

The intended purpose of variable selection is to identify a subset of predictor variables (i.e., the column of the **X** matrix in *Figure 2*) that can improve the performance of the model. The underlying rationale for variable selection in chemometrics is twofold. First, certain predictor variables could be irrelevant to the intended purpose, and ideally, these variables should be minimized or removed from the model. For example, only specific wavelengths of the whole range generated by the spectrometer may bear information relevant to the variation in levels of the response variable. Variable selection for this purpose should be based on first principles and experience. Inclusion of unrelated predictors, such as irrelevant spectral regions, could potentially degrade performance of the model. A smaller number of variables from the preprocessed data can be used to achieve superior performance, such as accuracy, precision, and robustness.

The second part of the rationale for variable selection is to avoid overfitting. Overfitting is the situation where the model is not only describing the intended variability in the data but also includes the modeling of the noise. The latter has a negative influence on the predictive properties of the model. Typically in chemometrics, the number of predictor variables (hundreds or thousands) is larger than the number of observations (dozens to hundreds). There are two general strategies for handling the issue of overfitting. One strategy is to manually or computationally select only a subset of predictor variables and use them for model development. Predictor variables can be selected manually (e.g., choosing certain spectral wavelengths characteristic of a given compound) or by using a variety of statistical selection methods such as stepwise regression and using simple univariate statistical measures such as the *F*-test, *t*-test, correlation coefficient, signal-to-noise ratios, intensity thresholds, variable importance in projection metric, or genetic algorithms. Multivariate approaches may use PC variable loadings, linear discriminants, or regression coefficients to define the key features.

In the second strategy for avoiding overfitting, variables are not selected at all. The latent variable methods—PLS and PCR—by their nature have the capability to selectively give more weight to the important predictor variables and de-weight the less important ones. These new latent variables, or components, are constructed as linear combinations of the observed variables. If combined with variable selection, the performance of latent variable models could potentially be improved, because some predictor variables contain only noise, thus perturbing the model-building process.

Selecting a subset of the predictor variables that are informative will reduce the size of the data set, provide computational efficiency, and obtain better models for postprocessing. One caveat for applications such as authentication is that eliminating predictor variables that are devoid of information may prevent the recognition of adulterants or novel objects that have features in these excluded predictor variables.

CROSS-VALIDATION

In practice, cross-validation is used to obtain an estimate of the model performance and to fine-tune an algorithm's parameters (e.g., the number of components for PLS). This is accomplished by repetitive splitting of the data (i.e., the number of rows of the **X** matrix in *Figure 2*) into a calibration set, which is used to develop the model, and a testing set (or internal validation set), which is used to make predictions and to compare actual and predicted values.

The approach of *n*-fold cross-validation is commonly used to split the data into *n* subsets to perform cross-validation. In each of the *n* iterations, *n*–1 subsets are used to develop the model, which is used to predict the remaining n^{th} data split. The procedure is repeated until all subsets are predicted. The error between reference values and predicted values during cross-validation is then recorded as root-mean-squared error of cross-validation (RMSECV). Multiple approaches exist for splitting the data into *n* segments. Regardless of how one decides to split the original calibration data, one aspect that deserves attention is that any batches used in calibration and cross-validation must not be considered or reused as an independent dataset for method validation. The most straightforward form is called leave-one-out cross-validation (LOOCV), where samples are removed one at a time. The LOOCV can involve intensive computations; its results can be heavily affected by outliers and are less consistent than the results of other forms of cross-validation. It is also possible to split the data according to prior information, such as one subset per batch given the available historical data set across multiple batches. Another option for cross-validation is bootstrapping, where in each iteration a certain proportion of data is randomly sampled to create a calibration set, while the remainder of the data are used as a testing set. The procedure is repeated many times (typically 100 or more cycles) to obtain a stable estimate of prediction error.

The measure of error is the most common figure of merit used to characterize model performance during cross-validation. The intended purpose of the method determines the nature of the errors, such as the misclassification rate for qualitative methods and prediction error for quantitative methods. Irrespective of qualitative or quantitative applications, two metrics are commonly used to characterize error within cross-validation: root-mean-squared error of calibration (RMSEC) and RMSECV. The RMSEC is calculated for the samples when left in the calibration, which monotonically decreases with each additional factor (i.e., PC) added into the model. In comparison, the RMSECV that is calculated during cross-validation will decrease until the last meaningful (i.e., relevant signal-containing) factor is added. Then, as each additional factor is incorporated into the model, the RMSECV will increase, indicating that the calibration data are being overfit. The plot of the RMSEC and/or the RMSECV versus the number of factors in the model is referred to as a predicted residual error sum-of-squares plot. In general, the best practice is to avoid inclusion of factors beyond where the minimum of the RMSECV plot line occurs. In addition, correlation between observed and predicted values (e.g., R^2) is also commonly used to assess performance of quantitative methods. Finally, the cross-validating results are only meaningful when the calibration and testing sets are comparable (i.e., drawn from the same population). Otherwise, extrapolation may lead to incorrect predictions.

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3.2 Method Validation

The objective of validation is to demonstrate that the performance of a method is suitable for its intended purpose. Validation of a model and validation of a method are two different activities. Model validation routinely involves the use of an internal validation set or cross-validation to assess the appropriate parameters of a model via identification or quantification error and uncertainty. Parameters often include the range of variables, the type of preprocessing, the model rank, the choice of the algorithm, and others. These activities have been addressed in detail in *3.1 Model Development: Calibration*. In comparison, method validation must be based upon a fully independent external validation set and must follow the validation requirement described in *Validation of Compendial Procedures* ⟨1225⟩, according to the method type category. The acceptance criteria should be justified for the intended purpose. During the lifecycle, method revalidation is necessary after a model transfer or model update. The method validation and revalidation strategy should be risk- and science-based and appropriate to its impact level.

The typical performance characteristics for method validation are specificity, accuracy, precision, linearity, range, and robustness for quantitative models, and specificity and robustness for qualitative models. The metrics and their descriptions are discussed below. In addition to those typical performance metrics, metrics such as limit of detection (LOD), limit of quantitation (LOQ), sensitivity, analytical sensitivity, and effective resolution may not be required for validation purposes but could be useful for understanding the boundary of the method performance for a specific analytical application and the analytical technique that a model is associated with.

PERFORMANCE CHARACTERISTICS FOR METHOD VALIDATION

The sections that follow provide method validation attribute descriptions and metrics.

Accuracy: Statistical comparison between predicted and reference values is recommended. For quantitative applications, root mean-squared error of prediction (RMSEP), squared error of prediction (SEP), and bias are the typical measures of method accuracy. For qualitative applications such as classification, sometimes misclassification rate or positive prediction rate could be used to characterize method accuracy. To ensure that a model is accurate enough when tested with an independent test set, RMSEP is often found to be comparable to RMSEC and RMSECV and to meet the requirements of ATP.

Precision: The routine metrics of RMSEP and SEP encompass both accuracy and precision. Assessment of precision could involve a determination of the uncertainty associated with the reportable result and a variance component analysis that determines an "error budget" that quantifies the sources of variation that contribute to the uncertainty. For an estimate of precision alone, the standard deviation across results for replicate and independent analyses on the same sample within method, across days, across analysts, across instruments, and across laboratories could be used.

Specificity: For both qualitative and quantitative methods, whenever possible the underlying chemical or physical meaning of the chemometric model should be demonstrated and validated. For example, the scientific meaning of the variables (such as spectral range) used for model construction, data preprocessing, regression vectors, and loading vectors [for PLS, PCR, PLS discriminant analysis (PLS-DA), and PCA] should be demonstrated. Specificity could also be validated by the tendency of sample components (matrix or other nonanalyte compounds present in the sample) to adversely affect the ability of the chemometric method to report results accurately and/or precisely. The level of specificity may be assessed by accuracy and precision in the presence of varying amounts of potentially interfering substances. Substances to be tested can be identified by considering the underlying physical/chemical methodology and modeling approach.

Specificity may be evaluated by including adulterated, substandard, or nonauthentic samples. Authentic samples, both within and out of the target criteria, may not be available for practical or economic reasons. For example, it may not be possible, due to cost, to obtain out-of-target samples for a controlled, large-scale process. Where possible, an exclusion panel of out-of-target samples that may be closely related to the target samples should be considered to increase the confidence in the specificity of the measurement. Simulated off-target samples (such as small-scale samples) may be used to validate the procedure's suitability for the intended purpose and range. In all cases, the suitability of validation samples must be justified with appropriate inclusion and exclusion criteria.

For qualitative methods, the method should demonstrate the capability to correctly identify or classify the samples. The receiver operating characteristic (ROC) curve and/or the probability of identification (POI) are commonly used metrics (detailed information on ROC curves can be found in *4.1 Qualitative* and *Figure 6*). The ROC approach is intended to illustrate true-positive rate (TPR) and false-positive rate (FPR) over a range of decision thresholds. A good identification method should generate a ROC curve with the area under the curve (AUC) close to 1. For most well-designed identification methods, the probability of a positive identification is near zero when the analyte is not present, and the probability should approach 1 as the analyte concentration or mass increases.

Linearity: The algorithm used for chemometric method construction can be linear or nonlinear, as long as it is appropriate for modeling the relationship between the analytical signal and the analyte. The measures commonly used to assess either the model fit or its predictive properties are the correlation coefficient, slope, *y*-intercept, and residual sum of squares of the plot between the predicted versus observed results. Note that the plot between the residual versus observed results across the analytical range is expected to show no pattern.

Range: The range of a method should be appropriate for its intended use (e.g., specification).

Robustness: Typical factors to be considered include the normal variability of materials (e.g., lot-to-lot variability), operating environment variability, instrument variability, such as minor instrument maintenance, and method parameter variability, such as the number of spectra averaged in a spectrometer. In conjunction with the validation results, the method development strategy—such as the design of the calibration set or library and the choice of model parameters—can be taken into account to demonstrate the method robustness.

VALIDATION SAMPLES

Other general aspects to be considered for chemometric method validation include the validation samples. The validation samples should be independent of the calibration samples to demonstrate the ability of the model to predict. Being independent

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means that the validation samples were not used in the calibration set or used for model optimization. Internal validation or cross-validation is typically used during calibration for model parameter optimization, but is not considered sufficient for final method validation. The validation samples should be selected based upon the ATP and the desired performance characteristics of the model. Method robustness is based on the evaluation of authentic samples with typical sources of variance. For pharmaceutical or dietary supplement products, validation samples of nominal production scale, such as those routinely manufactured for in-process, release, and/or stability testing, may be included. For botanical articles, taxonomic identity, geographic origin, season of collection, and other variants may be included. For naturally sourced materials such as many food ingredients and excipients, variables such as the geographic or microbiological source of the material, processing conditions, impurity composition, and other relevant attributes may be included in the validation sample set.

ACCEPTANCE AND DIAGNOSTIC CRITERIA

As with any other analytical procedure, the acceptance criteria for a chemometric method should be defined before execution of the validation protocol. If the chemometric model was developed using data from a secondary technique [e.g., near-infrared (NIR) or Raman] with reference values from a primary analytical procedure [e.g., gravimetric, nuclear magnetic resonance (NMR) spectroscopy, or high-performance liquid chromatography (HPLC)], the ATP for validation may not exceed that which is obtainable by the primary method. Some exceptions may occur, for example, it may be possible to achieve superior precision using a secondary procedure, although the accuracy will be limited to that of the reference technique.

In addition to those attributes addressed in *Performance Characteristics for Method Validation*, method validation must also take into consideration the setting of diagnostics limits for a multivariate model before deployment for routine use. Samples that are out of model space are considered outliers and not suitable for obtaining a reportable model prediction. The model diagnostic should have a demonstrated capability to flag any of the out-of-model-space samples. To set up the diagnostics limits, two cases must be considered. The first is to determine the statistical distribution of leverage and residual within-the-calibration data set. The second is to prepare intended in- and out-of-model-space validation samples to test the limit. For instance, for an NIR spectroscopy-based content uniformity method, the in- versus out-of-model-space samples could be samples at target label claim versus samples containing active pharmaceutical ingredient (API) concentrations outside of the intended range for the method.

3.3 Model Monitoring

Throughout the model lifecycle, changes that can affect model performance may occur. Procedures should be in place for continuous performance verification of the model and for model update and procedure revalidation if necessary. A specific order among model monitoring, model update, and model transfer does not exist. Scientifically sound rationales must be applied to determine an appropriate sequence for the intended application.

A control strategy for checking model performance over its lifecycle should be developed and documented as part of model development and procedure validation. The strategy should identify the necessary elements for ongoing monitoring and evaluation of model performance. In addition, a plan for monitoring, analyzing, and adjusting the model should be in place with a measurement frequency that allows identification of excursions related to critical aspects of the model. The level of maintenance should be considered part of the risk assessment activities and should be adequate for the model criticality. If applicable, analytical instrumentation used to generate the inputs for the model should be qualified and also subjected to a continuous verification plan (for relevant guidance, see general chapters related to the applicable analytical instrumentation).

Ongoing assurance of performance of the model throughout its lifecycle should include:

- Ongoing monitoring and review of the model
- Evaluation of risk assessment
- Evaluation of post-implementation changes and predefined model maintenance
- Model update and procedure revalidation as needed

The ongoing review of a model should occur at predefined intervals and also should be triggered by events that may have an impact on model performance. Examples of such events include changes in raw materials variability or manufacturer; changes in the upstream process that may alter the sample matrix (e.g., process equipment or operation settings); drifts in model prediction; and out-of-specification (OOS) or out-of-trend (OOT) results given by the model (dependent upon the root cause of the OOS or OOT). In addition to triggers that are based on the prediction output of the model, triggers based on model diagnostics metrics and corresponding action rules should be included. Multivariate models may be more strongly affected by aberrant data signatures than are univariate models. Special care is needed to: 1) justify the multivariate model diagnostics statistically, 2) verify with data from the model development and procedure validation process, and 3) implement multivariate diagnostics for monitoring as part of the control strategy. Comparison of model predictions and reference or orthogonal procedures should take place on a periodic basis or as part of the investigation triggered by the review process.

The use of model diagnostics when applied to a new sample ensures that the model prediction is valid with regard to the calibration and validation sets used during model development and procedure validation, and also ensures that the result does not constitute an outlier. The observation of an outlier means that the result is invalid, but it is not a reliable indication of an OOS result; an OOS result is an observation produced by a model when the prediction falls outside the acceptance criteria and the model diagnostics are within the thresholds. In the case of qualitative models, nonconforming results should be treated as outliers and should trigger an investigation; the output of such an investigation will indicate whether the result is OOS.

The review process for a model should produce a decision regarding the need for an extension of model maintenance; such a decision may be the result of a risk-assessment exercise or the outcome of a predefined decision workflow. Model criticality and usage will define the extension of model maintenance, which can include restraining the model conditions; adjusting the calibration set (samples can be added, replaced, or removed); or even completely rebuilding the model. The decision, and corresponding rationale, must be scientifically sound and documented.

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3.4 Model Update and Method Transfer

Model updating must be considered part of the analytical procedure verification, and both the justification and the activities must be documented as a part of the analytical procedure lifecycle. Before performing a model update, it is critical to understand the underlying causal factor that has prompted the update. The reasons for model updating can be roughly divided into two categories.

The first category is when the calibration set simply needs to be expanded. In this case, nothing has actually changed in terms of the response of the instrument to specific analytes. Instead, the original model is no longer valid because of the expanded range of original calibration, the addition of new analytes, or the occurrence of other, previously unseen variations (e.g., changes in particle size distribution, or the drift of a chemical process to a new steady state). Thus, the calibration space must be expanded with samples exhibiting this variation.

The second category is when the samples are the same but the measurement system response function has changed. This is often due to changes in the measurement components (new light source, clouding of optics, wavelength registration shift) or can be due to method transfer across different instruments. This, in essence, is an instrument standardization problem. Changes in instrument or measurement procedures over time can render a calibration model unusable, in which case a model update becomes necessary or a new model should be developed.

In practice, there are multiple model updating techniques that could be applied to each category of model update. Some updating techniques are relatively straightforward and simple to implement, whereas others are technically complex. Selection of an appropriate model updating approach must be based on a full understanding of the underlying causal factor. As a general rule, simple updating methods (e.g., slope and bias adjustment, as described below) should be considered first.

Before any model maintenance work is initiated, it is good practice to confirm that the fundamental construct of the original model—such as data preprocessing and variable selection—remains sound. For both qualitative and quantitative applications-based model updates, a straightforward preprocessing approach and a scientifically sound variable selection approach are recommended as the initial attempt to address challenges associated with updating the model. These approaches are equally applicable to differences caused by instruments, measurement conditions, or sample matrices.

SLOPE AND BIAS ADJUSTMENT

One of the simplest model-updating methods is to postprocess the predictions with a bias adjustment, a slope adjustment, or both. This approach is often used for quantitative applications. For some qualitative applications, this approach may also be useful, depending on the nature of the method. In a limited set of circumstances, bias/slope adjustments are expected to work. For example, if the matrix of the samples being predicted were systematically different from the calibration/validation sample set, the model predictions would be in error by a constant value. Bias/slope adjustments, however, would not correct for any new variation in the data, such as variation that would result from a different matrix (e.g., new analytes/interferents). Therefore, applying slope/bias adjustments without a full understanding of the underlying causal factor is not recommended. Guidance may be obtained via inspection of residuals corresponding to the new data obtained using the existing model or, alternately, from supplemental exploratory data analyses using techniques such as PCA (see *4. Applications of Chemometrics*).

CALIBRATION EXPANSION

When expanding the original calibration set, one should consider the number of new samples to be added, the impact (leverage) of the added samples on the overall composition of the new calibration set, and how to partition any new samples between the calibration and validation sets. It may be appropriate to simply augment the original calibration and validation sets with all the new samples, or it may be advisable to use a subset of the new and original samples. Multiple approaches, such as the Kennard-Stone algorithm and nearest-neighbor approach, are available to aid in the selection of new samples to add to an existing calibration set, but in general, all the approaches use methods to identify new samples on the basis of high leverage (i.e., model influence). Hotelling's T^2, multivariate score plots, and their corresponding limits are also effective approaches for accomplishing this goal.

CALIBRATION TRANSFER

Instrument standardization and calibration transfer methods are used to transform the response function of a measurement system so that it matches that of a reference measurement system. The reference measurement system could consist of a completely different analyzer, or it could be the same analyzer before it experienced a response-function shift. The vast majority of these methods generally requires the use of stable transfer samples that are measured on the original instrument and require the instrument to be standardized at the same time. In addition, the approaches commonly used for instrument standardization could also be applied effectively to address the challenges resulting from changes in sample matrix and measurement conditions.

3.5 Revalidation

Before the redeployment of a multivariate model, appropriate procedure revalidation should be established using criteria equivalent to those used in the original validation protocol. This revalidation is necessary to document the validity of the model as part of the analytical procedure verification. The nature and extent of the revalidation procedure, including aspects such as scientific justification and experimental approaches, must be based on the cause of the update and the nature of the corrective action required for establishing suitable performance. Revalidation should be documented as part of the analytical procedure lifecycle.

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4. APPLICATIONS OF CHEMOMETRICS

As discussed in *2. What is Chemometrics?*, chemometric analyses may be performed in either a supervised or unsupervised manner depending on the availability of data and the specifics of a given application. This section provides an explanation of these different analysis scenarios, as well as the chemometric tools (i.e., algorithms) that are commonly used. Additionally, several specific applications will be described in detail.

4.1 Qualitative

GENERAL ASPECTS

Qualitative chemometric analyses may be performed by using supervised and/or unsupervised approaches. However, to be incorporated into analytical procedures that are alternatives to compendial methods, the performance of any chemometric model must be verified as described in *3. Model Lifecycle*. This may not be possible if very little is known about the samples being analyzed. Nevertheless, unsupervised analyses play an important supporting role during the development of chemometric alternatives to compendial procedures within the lifecycle framework, and are recommended for use before the development of subsequent supervised approaches (examples are provided in *Qualitative Application Examples*).

Qualitative compendial procedures are those that seek to supply the value of some categorical descriptor for an unknown sample. Examples of categorical properties include (among others):

- Chemical identity: microcrystalline cellulose versus API
- Morphology: polymorph A versus B; monoclinic versus triclinic
- Sample authenticity: authentic versus nonauthentic and/or adulterated
- Sample origin: facility A versus B; lot ABC-001 versus lot XYZ-002

Categorical properties can be modeled effectively using supervised chemometric algorithms that leverage either proximity or distance in multivariate space, resulting in a qualitative assignment of samples to one or more classes depending on the application and the technique used. According to ⟨1225⟩, procedures based on models of this type are suitable for incorporation into Category IV methods for identification and should be validated to demonstrate adequate specificity for the intended use. "Identification" is a term that is generally used to describe a range of analysis scenarios; several common ones are described in *Qualitative Tools*.

Supervised techniques used for classification purposes, besides the analytical sensor information, make use of discrete information related to the samples in the data set (e.g., class labels). It is intended to define the borders of the clusters (or classes), providing statistical criteria, in addition to the visualization techniques (e.g., scores plot of PCs from a PCA). The borders then can be used as acceptance criteria for inclusion or exclusion of new samples into a given class. Several useful tools are available to the analyst for building the model, such as linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA), soft independent modeling of class analogy (SIMCA), kNN, and PLS-DA. More complex techniques also may be used with justification. Each type of model has its own strengths and weaknesses. If a particular algorithm does not give the desired level of performance, a different algorithm may be selected. These aspects are discussed further in *Qualitative Tools*.

For model development, optimization, and validation, the classification and discrimination techniques follow the same process described in *3. Model Lifecycle*. These techniques develop a threshold or limits to make class assignments. The simplest form of classification is a two-class system (e.g., good/bad or A/B). When a new sample is classified as good or bad (A or B) by the model, this results from what is called a discrimination technique. Classification is the technique of assigning a new sample as A, B, either A or B, or neither A nor B. The error rate of the model is the total number of incorrect classifications divided by the total number of samples. Depending on the requirements of the application and the associated risk levels, the error rate(s) might be class-specific or pooled across classes. The specific details of the application will determine whether the assignment of a sample to more than one class would be an acceptable result. Where this must be avoided, classification thresholds should be set appropriately during model development to guarantee single-class results.

QUALITATIVE TOOLS

PCA: PCA is a commonly used exploratory analysis tool that was briefly introduced in *2. What is Chemometrics?*. PCA is a variable reduction technique and acts on the data by defining new variables, so-called principal components (PCs, see *Figure 2*). PCs are orthogonal and are defined in the direction of the largest (remaining) variance in the data. The results of PCA are discussed in terms of PC scores and loadings, which may be plotted graphically in two (and sometimes three) dimensions for visualizing clusters of samples or outlying samples (score plots, see *Figure 4*), whereas the loadings plots provide information on the original variables. The scores are the projections of the samples on the PCs, whereas the loadings provide the weights (coefficients) by which the values of the original variables are multiplied to generate the component score for a particular sample. Loadings are rich in information regarding which variables in the matrix are prominent and can be used to "understand" the information captured by the latent variables that provide the basis for the observed sample distribution in the scores plot (*Figure 4*). The PCs may also be used as the basis for quantitative models via PCR (see *4.2 Quantitative*).

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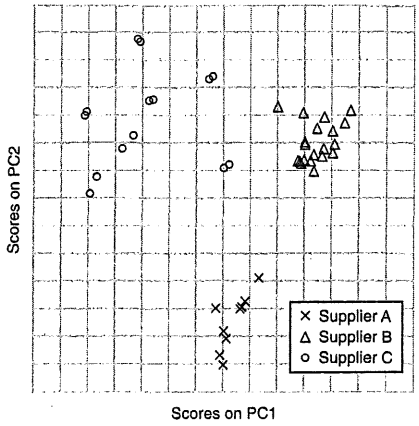

Figure 4. PCA scores plot: projection of PC1 versus PC2.

Clustering algorithms: The aim of clustering analyses is to partition data into different groups. Some methods, such as hierarchical cluster analysis (HCA), result in tree-like structures called dendrograms (*Figure 5*) that provide information on groups of samples or outlying objects occurring in the data set. Dendrograms can be built in many different ways and thus may show different aspects of the data set. A first possibility, called divisive or top-down methods, starts from the entire data set, which is split in consecutive steps until each object is in an individual cluster or all elements of a cluster fulfill a given similarity criterion. In the second scenario, called agglomerative or bottom-up methods, the opposite is done. Starting from individual objects/clusters, those most similar are merged until everything is in one cluster. Of the two options, bottom-up approaches tend to be computationally less intensive, are part of most computer packages, and are more frequently used.

The parameters used to express (dis)similarity between objects or clusters (see *y*-axis on *Figure 5*) can be either correlation-based (e.g., correlation coefficient, r for similarity or $1-|r|$ for dissimilarity), distance-based (e.g., Euclidian distance) measures, or a combination of both (e.g., Mahalanobis distance). Two clusters are linked in the dendrogram at the height related to this (dis)similarity measure. The parameter applied is expressed in such a way that clusters/objects linked low (i.e., close to the *x*-axis) are similar, whereas those linked high are dissimilar. Many different methods or criteria exist for use in deciding which objects/clusters are consecutively merged [e.g., single linkage, complete linkage, (weighted) average linkage, and centroid linkage]. Depending on the applied criterion, the dendrograms may look very different. Drawing arbitrary horizontal lines (see *Figure 5*) splits the data set into different groups, which occasionally may be linked to sample properties. Outlying samples are linked very high in the dendrograms, most often as one-object clusters. The stability of clustering results can be affected by many factors, such as noise in the data, sample size, choice of algorithm, distance measure, and others. Divisive methods tend to produce more stable results than agglomerative methods, even though they are hardly ever used.

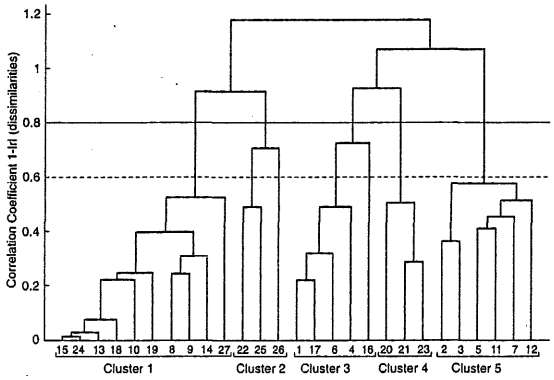

Figure 5. Hierarchical clustering based dendrogram for 27 objects. Abscissa: object numbers.

Parametric modeling: Authentication of a material or formulated product for quality assurance purposes may be achieved via multivariate parametric modeling of the chemical profile (e.g., spectrum or chromatogram). These methods are sometimes referred to as "one-class classifiers". Basically, a parametric model is developed statistically or empirically with a confidence boundary around the data from known authentic samples. Parametric models describe the behavior or structure of the majority of the data from a single class, assuming that a distribution (typically, a multivariate normal one) exists in those data. Predictions against a parametric model identify deviating points outside the significant boundaries of that distribution. Whereas classification or discrimination approaches provide identification (as previously described) of the unknown sample, parametric models will designate the unknown sample as either belonging to the authentic class or as an outlier, or exhibiting features other than those defined by the distribution. Some discrimination techniques will also optimize class separation, and may have greater selectivity, but will tend to fail when presented with an object from none of the classes. In contrast, parametric modeling methods will reject any sample that does not fall within the confidence boundary established for the model. Perhaps the most commonly used parametric modeling algorithm is SIMCA, where PCA is used to model the data and Hotelling's T^2 and Q

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residuals (DmodX) are used to define the limits. In some instances, parametric modeling approaches will result in "soft classifications" wherein a sample may be assigned to more than one class. To satisfy compendial usage requirements, appropriate action must be predefined for cases of ambiguous class assignment, and/or the α and β error rates must be established and justified according to risk.

QUALITATIVE APPLICATION EXAMPLES

Exploratory analysis: Unsupervised algorithms are routinely used for initial exploratory data analyses, which precede formal calibration and validation exercises. Unsupervised data exploration can rapidly indicate, in the absence of any prior knowledge, whether distinct classes or outliers exist within the pool of available data. For instance, in *Figure 4* a graphical example is shown in which PCA applied to spectroscopic data was used to differentiate between samples from different suppliers. Exploratory models yield latent variables that may be inspected to identify the original variables with the greatest amounts of relevant analyte signal to carry forward into subsequent models or to guide and/or verify the results of variable selection approaches. This might occur during the analysis phase of a DoE study to assess which factors to include in subsequent designs and/or calibrations. Further, exploratory analyses may be used to test and empirically optimize various signal preprocessing options to maximize the relative contribution of desired analyte signal versus signals from sources of interference (e.g., scattering effects, optical path length differences, sample physical properties, and instrumental drift).

Change to read:

Material identity testing: Identification testing is routinely performed for all pharmaceutical active ingredients, excipients, drug products, and packaging materials to authenticate them and verify that the correct materials are used in manufacturing, release testing, and packaging operations. ▲*Spectroscopic Identification Tests* ⟨197⟩▲ (CN 1-May-2020) illustrates one of the most widely used analytical techniques for identification, Fourier transform IR spectroscopy. Typically, identification testing involves comparing the sample spectrum to the spectrum of a reference sample and assessing the level of agreement between the two. Different chemometric algorithms can be applied for identification, and the general model development and maintenance should follow the guidance in *3. Model Lifecycle*. All methods for identity testing are supervised classification methods because either a single reference spectrum or a set of reference spectra from material(s) with known identity is utilized by these algorithms. Algorithms can be applied in either original variable space or transformed variable space (such as latent variable space) after data preprocessing.

According to ⟨1225⟩, models of this type are suitable for incorporation into Category IV methods for identification and should, at a minimum, be validated to demonstrate adequate specificity for the intended use. An example approach for performance verification is the documentation of TPR and FPR. TPR is the percentage of samples that are correctly identified, whereas FPR is the percentage of samples that are incorrectly identified. TPR is also referred to as sensitivity, and FPR is referred to as specificity. Regardless of which approach is taken, there is always a trade-off between FPR and TPR, and this trade-off behavior and overall procedure performance are best visualized in a ROC curve plot (see *Figure 6*).

A classical ROC curve is generated by plotting the TPR on the ordinate and the FPR on the abscissa. Each point on a ROC curve represents a specific TPR and FPR pair obtained at a specific threshold. The threshold is typically a number generated by the algorithm, and when it is exceeded, this corresponds to a positive identification. This threshold value is determined during the procedure development process. The threshold could be the *P* value from hypothesis testing, hit quality index (HQI) threshold, Mahalanobis distance threshold, PLS discriminant score threshold, or some other threshold.

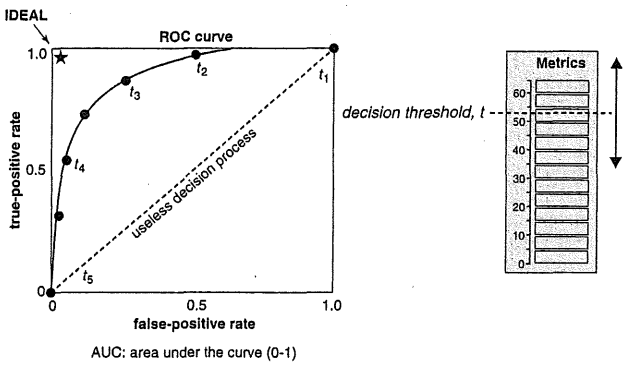

Figure 6. ROC curve plot showing the AUC (*3*).

During procedure development, a number of known positive and negative samples are examined, and the algorithm output for each sample is recorded. The performance of the method when operating at a particular threshold *t* is characterized by a single TPR/FPR pair, and the full operating characteristic curve can be produced by plotting TPR/FPR pairs over a range of decision thresholds. A false-positive identification corresponds to a decision threshold that is too low. False positives can be minimized by raising the decision threshold. However, excessively high decision thresholds can produce a false-negative identification.

A good identification method should generate a ROC curve that produces one or more TPR/FPR pairs in the top-left corner of the plot, as shown in *Figure 6*. This characteristic is best captured by the AUC. A random decision method is represented by the diagonal line with an AUC of 0.5, whereas a good identification method should have an AUC closer to 1. The shape of ROC

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curve depends upon a combination of spectral properties of the sample and the chemometric algorithm used for identification. Characterization of the ROC curve is a fundamental task associated with method development and validation.

For a two-class model, false positives and false negatives are the incorrectly assigned samples. Together with the true positives and true negatives, they provide the basis for the sensitivity and specificity of the model. The analyst may wish to care about one type of error (α or β) more than another, and therefore could change the model to be more conservative when choosing a threshold. Thresholds can be used as action criteria or evaluation criteria.

Complex material authentication testing: Nutraceuticals and samples of herbal origin may be complex mixtures of numerous components. Authentication relies upon assessment of the presence of multiple analytes and their relative concentrations as well as potentially demonstrating the absence of adulterants and/or related materials. Thus, authentication in this scenario is a multivariate decision process that is appropriate for chemometric approaches. Typically, a characteristic "fingerprint" pattern incorporating information from the various analytes (and adulterants, potentially) that are present in the sample is obtained by applying a combination of separation and characterization techniques or spectroscopic measurements. The chemical composition information in the sample fingerprint provides a basis for multivariate pattern recognition. In particular, techniques with exceptional analytical selectivity, such as mass spectrometry and NMR spectroscopy, have emerged as powerful fingerprinting tools, especially when operated as detectors for chromatographic separations of multicomponent samples. Chromatographic fingerprinting is recognized as a viable identification procedure for herbal medicines by both the World Health Organization and the U.S. Food and Drug Administration. (*4*,*5*)

Depending on the origin and purity of the sample, it may be appropriate to develop a multivariate classification model using either the entire fingerprint or a selected subset of peaks. Variable selection approaches may be useful in determining the optimal approach, depending on the nature of the samples and/or the ATP. It is crucial that the samples have an associated class label or quantitative property (activity) obtained by an objective reference technique (separate from that used to generate the fingerprint) to enable supervised modeling and subsequent model performance verification. Care should be taken to ensure that appropriate sources and levels of variability are included in model development and performance verification exercises based on the intended use of the test.

Several useful tools are available to the analyst for building the model, such as LDA and QDA, SIMCA, kNN, and PLS-DA. More complex techniques may also be used with justification (e.g., SVM).

Chemometric methods for multicomponent sample authentication are consistent with *Validation of Compendial Procedures* ⟨1225⟩, *Validation, Data Elements Required for Validation, Category IV*. Various numerical methods may be used to verify predictive performance. As discussed in *Qualitative Tools* for pure material identification, predictive performance may be verified by comparison of the TPR and FPR once a classification threshold appropriate to the application has been established. It is crucial to finalize this threshold before formal validation of the overall analytical method.

Classification: This is a supervised learning technique. The goal of calibration is to construct a classification rule based on a calibration set where both **X** and **Y** (class labels) are known. Once obtained, the classification rule can then be used for the prediction of new objects whose **X** data are available.

Classification models may be divided into two main groups: hard and soft. Hard classification directly targets the classification decision boundary without producing a probability estimation. Examples of hard classification techniques are kNN and expert systems. Soft classification estimates the class conditional likelihood explicitly and then makes the class assignment based on the largest estimated likelihood. SIMCA is a commonly used soft classification technique. In either scenario, the thresholds to determine the class boundaries are important and should be established during method development based on the resulting TPR and FPR, the requirements for which are determined by the ATP. These rates should also be verified as part of the full procedure validation.

In many applications, it is unacceptable to have a sample classified into two classes. Further, if a sample is not classified into any class, the sample is deemed an outlier to the calibration set. For example, a classification model built from NIR spectra of four materials (A, B, C, D) has four classes. Any material that has a source of variance (e.g., different water content) not previously seen and included in the model calibration may not be classified, and therefore should be identified as an outlier and investigated. Similarly, when a spectrum of another material (E) is applied to the model, the model should not be able to classify it into a given class. If similar samples are expected in the future, then the model should be revised to include the outlier samples in the calibration set, either to supplement the calibration data for existing classes or to create an entirely new class, depending on the nature of the outlier(s) and the specific requirements of the application.

4.2 Quantitative

GENERAL ASPECTS

When the intended use of an analytical procedure is the determination of one or more sample components or properties that have values that can vary continuously rather than categorically, then a quantitative model is required. Similar to the process for qualitative modeling, quantitative chemometric models are produced in a supervised manner using both the independent **X**-block and dependent **y** variables to ensure optimal predictive performance. The difference arises in the nature of the **y** values, which are continuous/numerical (rather than categorical) for quantitative applications. Per ⟨1225⟩, quantitative chemometric models are suitable for incorporation into Category I, II, or III methods for determination of the content of drug substance or finished drug product, impurity levels, or performance characteristics, respectively. In-process tests applied in an offline manner or an in/on/at-line manner that produce the value for a continuous variable would also represent appropriate uses of quantitative chemometric models. The specific requirements of the analysis per the ATP (see *3. Model Lifecycle*) should be used to guide the validation metrics used to demonstrate performance.

QUANTITATIVE TOOLS

The algorithmic tools needed for quantitative applications are wide ranging; for more complete descriptions see *Appendix, Additional Sources of Information*. Tools that are commonly used are briefly described here. As is the case with any chemometric

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model, the tool must be matched to the ATP, and vice versa. Certain advantages and disadvantages are characteristic of each tool, and the chemometrician must be mindful of these when justifying their use.

Multiple Linear Regression (MLR): This tool establishes a correlation between a dependent variable (or **y**, response variable) and multiple independent variables (or **x**, explanatory variables) by fitting a linear equation to observed data. MLR has two broad applications, as follows.

The first broad application of MLR is to establish a model that explains the importance or magnitude of effect on the response variable, and this is typically used in the DoE screening or response analysis. Typically the input variables are mutually independent and by design represent large magnitudes. Often, the higher order of terms such as square terms and interaction terms are included, and a step-wise procedure is used to exclude those that make a smaller contribution to the model. Caution is advised to avoid having too many variables in the model, because including too many terms will overfit the model. The adjusted R^2 statistic is typically used to estimate whether there are too many factors in the model, although other metrics may also be appropriate. Adjusted R^2 is a modification of the standard coefficient of determination that takes into account the number of model terms and the number of samples, resulting in a plot of R^2 (adjusted) that has a maximum value beyond which additional terms result in overfitting.

The second broad application of MLR is to establish a prediction model for analytical purpose. MLR can be used to fit a predictive model to an observed data set of **y** and **x** values. The established model can be used to predict the value **y** from the **x** of new samples. The model for prediction purpose created from correlated **x** inputs is suitable to explore the relationship between **y** and individual **x**'s.

The prediction MLR model can be used in a procedure or method for a pharmaceutical compendial test. For example, a filter NIR, which is designed for a specific use such as water testing in the product, produces a "spectrum" of only a few data points.

The development and validation can follow the same procedures as are used with latent variable models such as a PLS. During the development and validation of an MLR model: 1) appropriate data preprocessing needs to be used; 2) cross-validation is used to estimate the model performance; and 3) an independent data set is used to assess the model.

A limitation of MLR is that model predictive performance may be negatively impacted when the modeled variables are collinear. The issue of collinearity is not unique to MLR. It is mitigated in PLS modeling via generation of a new basis set of orthogonal (noncollinear) latent variables that are linear combinations of the original variables. However, this is not relevant for MLR models. In MLR models, variable selection or transformation (e.g., mean centering) may be needed to avoid or reduce collinearity in the calibration set. Even though MLR models have some diagnostic metrics to identify outliers and highly leveraged samples in the calibration, they do not have diagnostic metrics (such as Hotelling's T^2 and **X** residual test) that PLS models offer during the prediction of new samples.

PCR: This is a two-step modeling approach in which a PCA is first performed on the **X**-block data to obtain a basis set of PCs. The PCs are then used in combination with the **y** data to develop a regression model to predict the **y** values.

PLS: This is one of the most commonly used chemometric algorithms for both quantitative and qualitative (PLS-DA, see *Qualitative Tools*) modeling scenarios. The computational advantage that PLS offers over MLR and PCR is that the PCs are derived in the PLS algorithm using the **X** and **y** data simultaneously. This often results in predictive models that require fewer latent variables compared to PCR, thereby improving robustness. PLS is capable of handling mildly nonlinear relationships, whereas PCR and MLR are linear modeling techniques. Further, more than one **y** variable can be modeled using the same **X**-block data via different algorithmic approaches. PLS-1 results in a separate model for each **y** variable. PLS-2 will produce a single model capable of simultaneously predicting the values of two or more **y** variables. The relative advantages of each approach may vary from one application to another. For instance, if different mathematical preprocessing is necessary to resolve each component to be predicted, separate PLS-1 models may provide improved performance over a single PLS-2 model.

QUANTITATIVE APPLICATION EXAMPLES

Pharmaceutical dosage form assay and/or uniformity of content: Assay of the API content in the final dosage form is commonly performed via measurement of a sample drawn from a homogenous composite of a number of individual dosage units (e.g., tablets or capsules). The quantitative result is then reported as an "average content" (expressed as percentage of target API amount) for the batch tested. Uniformity of content may be tested using a method similar to the assay, but measurements are performed on individual samples. The reported result is primarily based on the variability in assay results across the individual dosage units analyzed. Assay and uniformity of content methods are intended to characterize the "major" component in the sample(s) in question and are considered Category I tests per the definitions established in ⟨1225⟩. In the majority of cases, the target analyte will be the active ingredient in the sample, although quantitative assays for other components exist throughout the compendia as well.

For pharmaceutical solid dosage forms, the majority of assay procedures are based upon HPLC. HPLC analysis is time consuming and often involves the use of large volumes of solvent for the mobile phase. However, HPLC is highly linear and is typically calibrated using univariate mathematics (i.e., one measured variable is directly and uniquely proportional to sample concentration).

In contrast, alternative methods for assay that are based on spectroscopy (e.g., NIR and transmission Raman spectroscopies) offer the advantage of increased analysis speed and are nondestructive. However, in many cases the analyte signal may not display the same degree of linearity or signal-to-noise as with the corresponding HPLC method. Moreover, no single variable is directly and uniquely related to the concentration of interest. For this reason, multivariate models, often based on PLS regression, are commonly used in spectroscopic procedures for assay and uniformity of content.

For Category I tests, it is crucial to demonstrate the accuracy, precision, specificity, linearity, and range of results during the validation of the overall analytical procedure. Each of these performance aspects involves considerations during multivariate model development or validation that are distinct from those related to univariate techniques.

Most spectroscopic methods are not quantitative in an absolute sense. For example, models developed using NIR or Raman data must be calibrated relative to a primary reference technique (often HPLC or NMR spectroscopy). Given the nondestructive nature of NIR and Raman, it is straightforward to analyze a given set of samples using one of these techniques and then subsequently analyze the same set of samples via HPLC or NMR spectroscopy to obtain the reference content values. This

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approach is typical for assays of solid dosage forms, however, it is worth noting that reference values for assays of some dosage forms, APIs, polymorphs, or excipients might be derived from other techniques such as gravimetric data recorded during the calibration sample preparation. The chemometric models are developed via a multivariate calibration using the NIR or Raman spectral data and the content values from the reference technique. Accuracy is determined by using the absolute difference between the model predictions and the reference values. Precision is determined by using the standard deviation of procedure results alone, such as from replicate analyses of the same sample. Thus, precision is a measure of performance for the analytical procedure as a whole. The model itself will be absolutely precise. That is, given the same spectral input, the same numeric output will always be generated. That said, precision may be influenced by the specifics of the chemometric modeling approach (e.g., preprocessing, modeling algorithm, number of latent variables) and therefore precision should be evaluated during model development as well as during validation. It is important to note that the accuracy of chemometric models will not be able to exceed the accuracy of the reference analytical technique used to calibrate the model. However, it may in certain cases be possible to exceed the precision of the reference technique.

The range of the method will be determined by the calibration samples that are used to develop the multivariate model. According to ⟨1225⟩, it is recommended that assay calibration samples should have analyte contents ranging from 80%–120% of the target amount. For evaluating uniformity of content, the recommended range is 70%–130% of the target amount. In some cases, both the assay results and uniformity results may be obtained from the same set of sample measurements. Averaging of a requisite number of individual dosage-unit assay results will provide a value equivalent to the "average content" parameter. Calculation of the "acceptance value" (see *Uniformity of Dosage Units* ⟨905⟩) using this mean result combined with the standard deviation for the same set of individual dosage unit results will provide a uniformity of content result. In this scenario, a single calibration set that spans the wider of the two recommended ranges (70%–130%) should be used.

If it is not practical to obtain a sufficiently wide range of content values in calibration samples produced at commercial manufacturing scale, calibration samples may also be produced in a smaller scale or in the laboratory. In this scenario, all attempts should be made to replicate the physical properties representative of the commercial scale. Additionally, care should be taken to verify that minimal bias exists between samples from different scales. Commercial-scale samples may need to be incorporated into the model (and/or procedure) validation process to verify the accuracy and precision of the model (and/or procedure) results against any differences and/or variability in physical properties. Mathematical processing algorithms (e.g., normalization, second derivatives) should be optimized as much as possible during initial exploratory data analysis (see *Qualitative Application Examples*) to mitigate prediction bias resulting from these factors. For instance, it is known that thickness, particle size, and density differences can lead to spectral slope changes and baseline offsets in NIR spectra. Depending on which specific effect is present, improperly selected preprocessing may not fully correct for spectral differences, leading to errors in prediction.

Fit and predictive performance of chemometric models should be demonstrated via the linearity of a plot of the model outputs versus the reference or nominal values of the target analyte(s), ideally using results from a set of independent test samples. The raw analytical signal may demonstrate a nonlinear relationship with the reference analyte concentrations. Likewise, multiple latent variables may be utilized for the chemometric model building. However, the key aspect of the model that must be demonstrated to vary linearly with analyte concentration is the model outputs, not the inputs. A plot of the prediction residuals versus concentration may assist in revealing any systematic lack of fit. Any observations of patterns in this residuals plot may indicate a need for revision of either 1) the number of latent variables included in the model, 2) the preprocessing mathematics, or 3) the algorithm type used for modeling.

Demonstration of the specificity of the procedure for assay may be based on likely sources of interference or material substitution based on an understanding of the material properties of the sample components and the manufacturing process and/or supply chain. One approach might be to verify that low assay results that are OOS are consistently obtained for a placebo version of the dosage unit. Another approach might be to verify that dosage units of a different product (especially one manufactured in the same facility and/or tested in the same laboratory) result in assay values that are consistently OOS.

Impurity limit tests: Routine testing is required for determination of impurities and/or degradants in intermediates, bulk drug substances, and finished pharmaceutical products. Testing for this purpose may take the form of a limit test (Category II, ⟨1225⟩).

Typically, these impurities tests are based upon HPLC or other methods (e.g., Karl Fischer titration for water). These analytical procedures are highly linear and are typically calibrated using univariate mathematics. However, these methods are often time consuming and involve manual sampling, which is disadvantageous, especially for products having high potency or for those with toxic intermediates produced during API synthesis.

In contrast, alternative methods for assay based on spectroscopy (e.g., NIR and Raman spectroscopy) offer the advantage of increased analysis speed, and more importantly, total elimination of human sampling due to the noninvasive nature of the measurement. However, these advantages are typically offset by decreased sensitivity (LOD) and potentially nonlinear sensor response across the required analyte concentration range as the corresponding reference method. For this reason, multivariate approaches combining spectral preprocessing and latent variable models are often employed in spectroscopic methods for these limit tests. (*6*) However, due to the potential challenges involved in achieving adequate sensitivity for a spectroscopy-based limit test, careful development and feasibility studies must be employed.

For a limit test in Category II procedures, a calibration set containing varied concentrations of the analyte of interest is often used to characterize the performance of such an analytical procedure. Although it is not required, these performance metrics could include accuracy, range, linearity, and others. The use of the performance metrics depends on the nature of the limit test.

In contrast, it is critical to illustrate specificity and LOD during validation of a limit test according to ⟨1225⟩. For specificity, a comparison between loading/regression vector and a spectrum representative of the pure component of interest is often used. Additionally, measuring matrix effects on the determination of the analyte within the specified range is another useful approach to demonstrate that the analytical procedure is specific to the analyte of interest without impacts from other variables introduced in the calibration set. Moreover, evidence of method specificity can be demonstrated by calculation of a selectivity ratio from the calibration data set, defined as the variance of model-reconstructed spectra divided by that of residual spectra. The selectivity ratio at the analyte absorption band is expected to be higher relative to other spectral regions.

Regarding LOD, there is no generally accepted estimator for PLS models. A common practice to determine LOD involves calculating a spectral signal-to-noise ratio. The model results for replicate analyses of a blank sample(s) may be used to represent

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the noise. Alternatively, the noise may be approximated using the standard error of a multivariate model. Other more elaborate approaches were suggested to address the multivariate nature of PLS models. (*7*) With any approach, the estimated LOD should be verified experimentally using samples with analyte levels at or near that concentration. Because the LOD obtainable via a spectroscopic method will likely be higher than that obtainable via a chromatographic approach, it becomes crucial to demonstrate that the LOD based on the chemometric model meets the requirements of the ATP.

Dissolution testing: In vitro dissolution is a crucial performance test for solid dosage forms that is used to predict in vivo drug-release profiles during drug development and to assess batch-to-batch consistency for quality control purposes. Chemometric models can be used to predict the drug product dissolution from relevant measured product properties, and the method that uses such a model may be established as an alternative procedure. Alternatively, the dissolution profile can be modeled via several responses from different time points on the dissolution curve using individual models.

Dissolution data may be collected as a profile (i.e., a series of values obtained at various time points), even though the acceptance criteria may rely on a single time point. When building a chemometric model, it is essential for the model to be able to predict the entire profile. The number, and spacing in time, of points in the dissolution profile will vary depending upon the product type (e.g., immediate versus modified/extended release). A common approach is to transform the profile into a single value, which then serves as a dependent variable (or **y**, response variable). This variable, combined with another measured variable, can be used to restore the dissolution profile. The transformation can be accomplished by fitting the dissolution profile data into a mechanistic model (such as a first-order rate equation or its variants) or an empirical model (such as a Weibull function). After transformation, a few variables (typically, two or three variables) can be used to represent the dissolution profile. Generally, the two variables are a dissolution rate factor and a plateau factor; a third variable such as lag time may be necessary. The dissolution rate factor, which represents the rate of dissolution, will be the dependent variable for the modeling. The plateau factor represents the final amount of the drug in the solution, which should be equal to the drug content for most products. These two factors are used to restore the profile. Therefore, the model for dissolution prediction is used to predict the dissolution rate.

The key to designing a successful model is defining the input variables. The plateau factor may be obtained from drug content data, whereas the dissolution rate factor may be modeled from a data set of chemical and physical attributes of the product or intermediate product. The necessary knowledge of physicochemical properties and engineering concepts should be used to identify and justify the relevant inputs that have potential impact on the dissolution of the product. This typically involves risk assessment and an additional data collection step such as a small-scale DoE study. The inputs typically include the measured material attributes at various processing stages (such as particle size data for granules or blend; NIR measurements for blend or tablets; and physical tablet properties).

There are two approaches for defining samples in the model calibration: batch samples and individual unit dose samples. For the batch-sample approach, one product batch is treated as a sample, and the input variables are the attribute means of the batch. The individual unit dose approach is to measure the attributes of individual unit doses (such as NIR measurement on the individual tablets). The input variables from both approaches may be supplemented with other raw material properties as justified by the critical quality attributes.

The validation of the model-based dissolution prediction method could be different from the typical HPLC dissolution method validation, depending on the specific approach used. The HPLC dissolution method puts more emphasis on precision, as the dissolution is treated as a Category III method in ⟨1225⟩.

In the batch-sample approach, the model-based method treats dissolution as a batch property instead of a property of individual tablets, and accuracy is the focus of method validation or verification. For example, each batch forms a sample, and it is not realistic to have a large validation sample set. This approach generates a single profile for a batch, and the result at a certain time point can be used to assess the product batch quality. Evaluation of variability must be aligned with the criteria in *Dissolution* ⟨711⟩. The variability of the final dissolution result can be evaluated by analyzing the variations of inputs. Possible ways to evaluate the variation of the sample (batch) include the analysis of variation of inputs, simulations, and dividing a batch into multiple sub-batches.

In the individual unit dose approach, the model-based method evaluates individual dosage units. If many tablets are tested, this becomes a large *n* situation in which the acceptance criteria in ⟨711⟩ should be used with caution. The chemometrician may propose acceptance criteria as long as these criteria are demonstrated (by simulation or other means) to have equivalent or tighter passing criteria than those in ⟨711⟩. In the method validation or verification, it is desirable to have at least one sample that shows low dissolution (near or below the specification), and the chemometric model-based method should demonstrate the capability to distinguish this low-dissolution sample from normal samples.

GLOSSARY

Many common chemometric terms have been defined within the text of this chapter. Some additional terms are defined here and serve as a reference to their usage in the text. For a more complete description, consult the texts listed in *Appendix, Additional Sources of Information*.

Calibration model: A mathematical expression used to relate the response from an analytical instrument to the properties of samples, or to capture the underlying structure of a calibration data set.

Calibration set: Collection of data used to develop a chemometric classification or model.

Derivatives: The change in intensity with respect to the measurement variable (i.e., abscissa). Derivatives are useful for removing baseline offsets (constant or linear) due to sample properties, or for highlighting small changes in a signal, helping to enhance selectivity and sensitivity (e.g., one spectroscopy and chromatographic peak with a shoulder peak adjacent).

Internal validation: The application of resampling statistics such as cross-validation. Subsets of the calibration data set are subjected to a variety of statistical processes to identify which calibration model best fits the available data. Each model is characterized by a statistical parameter. For cross-validation, the entire data set of samples is split into individual samples or groups of samples, which are removed individually from the rest of the samples and tested as unknowns against a calibration model constructed using the rest of the samples. The characteristic statistic is the standard error of cross validation (SECV).

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Matrix: A two-dimensional data structure comprised of columns and rows used to organize inputs or outputs during chemometric analyses. Common practice is for each row to correspond to an individual sample and each column to correspond to an individual variable.

Validation set: The data set that challenges the performance attributes of the model. The validation data set is independent of the training data set, although testing on the training data set gives an optimistic view of performance and thus allows for iteration of preprocessing and model tweaking until this optimistic view meets the expectations. Validation or cross-validation is then necessary to adequately gauge the performance of the model. The model performance with the validation data set will always be equal to or worse than its performance with the training data set. If the performance attributes are not met, then the chemometrician must assess whether the model underfits or overfits the data and whether iterations of the model are needed to meet the acceptable error rate. If these efforts fail, it is recommended to back up one step, alter the preprocessing conditions, and perform the same task again.

APPENDIX

Additional Sources of Information

Many books have been written on the subject of chemometrics and multivariate analysis. Various terms not found in the glossary may be found in this short list of additional sources of information.

- Massart DL, Vandeginste BGM, Buydens LMC, De Jong P, Lewi PJ, Smeyers-Verbeke J, eds. *Handbook of Chemometrics and Qualimetrics: Part A.* 1st ed. Amsterdam: Elsevier Science B.V.; 1997.
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⟨1041⟩ BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations —Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.

Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only

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upon approval by the Center for Biologics Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see *Safety Tests—Biologicals* under *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Sterility Tests* ⟨71⟩, *Water Determination* ⟨921⟩, and *Pyrogen Test* ⟨151⟩, as well as *Bacterial Endotoxins Test* ⟨85⟩). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. (*General Notices, 5.50.10 Units of Potency (Biological)*.)

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of *USP Supplements* as promptly as practicable.

VEHICLES AND ADDED SUBSTANCES

Vehicles and added substances suitable for biologics are those named in the Food and Drug Regulations.

CONTAINERS FOR INJECTIONS

Containers for biologics intended to be administered by injection meet the requirements for *Packaging and Storage Requirements* ⟨659⟩, *Injection Packaging*.

CONTAINER CONTENT

The volumes in containers of biologics intended to be administered by injection meet the requirements for *Container Content for Injections* ⟨697⟩.

LABELING

Biologics intended to be administered by injection comply with the requirements for *Labeling* ⟨7⟩, *Labels and Labeling for Injectable Products*. In addition, the label on the final container for each biologic states the following: the title or proper name (the name under which the product is licensed under the Public Health Service Act); the name, address, and license number of the manufacturer; the lot number; the expiration date; and the recommended individual dose for multiple-dose containers. The package label includes all of the above, with the addition of the following: the preservative used and its amount; the number of containers, if more than one; the amount of product in the container; the recommended storage temperature; a statement, if necessary, that freezing is to be avoided; and such other information as the Food and Drug regulations may require.

PACKAGING AND STORAGE

The labeling gives the recommended storage temperature (see *Packaging and Storage Requirements* ⟨659⟩). Precautions should be taken where products labeled to be stored at a temperature between 2° and 8° are stored in a refrigerator, in order to assure that they will not be frozen. Diluents packaged with biologics should not be frozen. Some products (as defined in Section 600.15) are to be maintained during shipment at specified temperatures.

EXPIRATION DATE

For compendial articles the expiration date identifies the time during which the article may be expected to meet the requirements of the Pharmacopeial monograph, provided it is kept under the prescribed storage conditions. This date limits the time during which the product may be dispensed or used (*General Notices, 3.10 Applicability of Standards*). However, for biological products, the stated date on each lot determines the dating period, which begins on the date of manufacture (Section 610.50) and beyond which the product cannot be expected beyond reasonable doubt to yield its specific results and to retain the required safety, purity, and potency (Section 300.3 (l) and (m)). Such a dating period may comprise an in-house storage period during which it is permitted to be held under prescribed conditions in the manufacturer's storage, followed by a period after issue therefrom. The individual monographs usually indicate both the latter period and (in parentheses) the permissible in-house storage period. If the product is held in the manufacturer's storage for a longer period than that indicated (in parentheses), the expiration date is set so as to reduce the dating period after issue from the manufacturer's storage by a corresponding amount.

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⟨1043⟩ ANCILLARY MATERIALS FOR CELL, GENE, AND TISSUE-ENGINEERED PRODUCTS

Change to read:

INTRODUCTION

A wide variety of reagents and materials, many of which are unique or complex, are ▲used in▲ (USP 1-May-2020) the manufacture of cell, gene, and tissue-engineered ▲(CGT)▲ (USP 1-May-2020) products.

▲The purpose of this chapter is to provide guidance on the development of appropriate material qualification programs for CGT products. Because CGT products are not usually amenable to extensive purification, filtration, and terminal sterilization procedures, reagents and material qualification are critically important to ensuring CGT product quality.

The term "ancillary materials" (AMs) refers to materials that come into contact with the cellular starting material, CGT product intermediates, or final CGT product during manufacturing, but are not intended to be present in the final CGT product. AMs are therefore distinct from the cellular starting material, the intermediates, and final CGT products. Excipients, which are intended to be present in the final product, are therefore not AMs. Processing devices such as vessels and transfer tubing sets may be considered AMs if they have surfaces and materials that contact the cells during production. Processing devices and containers that are considered AMs typically incorporate natural or synthetic biomaterials. Scaffolds or delivery devices that are intended to be part of the final product are not AMs. Cell banks and virus banks are generally not considered AMs; feeder layer cells that are not intended to be incorporated in the final product may be considered an AM or an impurity. Guidance documents (i.e., ICH Q5D) describe specific requirements for cell and virus bank certification and regulatory approval. However, "helper" viruses and "helper" plasmids may be considered AMs if they are not intended to be part of the final product.

Reagents that fall under the AM classification include well-characterized chemicals, complex compounds (antibiotics, anticoagulants, density gradients, toxins), multi-component mixtures (buffers, culture media), and complex biological compounds or mixtures (enzymes; blood-, plasma-, or serum-derived products; biological extracts; cytokines; antibodies; and conditioned media from cultured cells).▲ (USP 1-May-2020)

Add the following:

▲REGULATORY CONSIDERATIONS

Regulatory agencies have referred to AMs (as defined within this chapter) as ancillary products, ancillary reagents, processing materials, processing aids, and process reagents. The US FDA first introduced the AM concept with the term "ancillary product" in a *Federal Register* notice, *Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products* [*Federal Register* 58(197), October 14, 1993, pp. 53248–53251], which established the FDA's authority to regulate human somatic cell therapy products and gene therapy products. In a subsequent guidance, the FDA defined the term "ancillary reagent" as a material "used in the manufacture or production of a biological product that may or may not end up as part of the final product," but the definition was not formalized or used consistently in other FDA guidance (see *Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications*, February 2010). The term "AM" is also synonymous with "processing material", as defined in 21 CFR 1271, *Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule* [*Federal Register* 66(5), January 8, 2001, pp. 1508–1559]. The US FDA also provides information to cell and tissue product manufacturers in 21 CFR 876.5885, *Tissue Culture Media for Human Ex Vivo Tissue and Cell Culture Processing Applications*.

Various labeling terminology has been used by AM manufacturers. The labeling terms "Research Use Only" (RUO) and "Investigational Use Only" (IUO) originated in the medical device labeling regulations and apply to materials intended for in vitro diagnostic use (see *Distribution of In Vitro Diagnostic Products Labeled for Research Use Only or Investigational Use Only: Guidance for Industry and FDA Staff*, November 2013). Because a given AM's label may or may not be relevant to its intended use in CGT product manufacturing, it is ultimately the responsibility of the CGT product manufacturer to define and qualify the suitability of a given AM in the CGT product manufacturing process.

Global and regional differences in AM labeling and documentation may dictate CGT product regulatory submission requirements. In the US, the CGT product sponsor can sometimes cross-reference an AM manufacturer's Drug Master File (DMF), which allows proprietary AM information to be reviewed by regulators, but not necessarily by the CGT product manufacturer. Other countries and regulatory authorities manage the submission of AM information differently, and it is critically important for the CGT product manufacturer to be aware of these differences if the same CGT product will be clinically tested or marketed in more than one regulatory jurisdiction. In any case, the CGT product manufacturer is responsible for ascertaining the AM's labeling, essential features, quality characteristics, and suitability for use.▲ (USP 1-May-2020)

Add the following:

▲IMPACT OF ANCILLARY MATERIAL QUALITY ON PRODUCT QUALITY

AM quality can pose risks to the stability, safety, potency, and purity of any product. These risks are often heightened with CGT products, whether due to their short shelf lives, limited ability to conduct extensive in-process and release testing, or the

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fact that many CGT products expand in vivo after their administration to the patient. To minimize the risks associated with these commonly occurring scenarios, rigorous AM qualification and prudent application of manufacturing process controls are necessary.

Sourcing of Ancillary Materials

AMs used in the manufacture of CGT products are often selected for their unique functional or biological effects, and not necessarily on their quality attributes. Many AMs are only intended for research use but may not be readily available in a grade comparable to that of well-characterized licensed therapeutic products manufactured by controlled and documented methods. For this reason, many CGT product manufacturers source AMs that are approved or licensed therapeutic products. More recently, many AM suppliers have committed to providing higher grade AMs, using high-quality manufacturing and testing approaches with extensive documentation. Although the terms "clinical grade" and "good manufacturing practices" (GMP) are often applied to these higher quality AMs, their documentation may not be fully compliant with regulatory requirements for finished pharmaceutical products and excipients. Regardless of the AM labeling claims or descriptions, CGT product manufacturers are ultimately responsible for ensuring that the AM meets the necessary functional, quality, and documentation requirements demanded by the relevant regulatory authorities.

Specific safeguards are necessary in order to minimize or eliminate the risk of transmitting adventitious agents (viruses, bacteria, mycoplasma, protozoa, fungi, prions, etc.) when sourcing AMs using human- or animal-derived components such as sera, antibodies, or growth factors. In general, manufacturers of human- or animal-derived components and AMs should practice the following procedures to ensure CGT product safety:

1. Screening and qualifying (e.g., through bacterial or viral testing) and documenting (e.g., through herd certification and traceability) the sources of human- or animal-derived components as being free of suspected adventitious agents
2. Validating the inactivation (e.g., through chemical, thermal, or irradiation treatments) and removal (e.g., through chromatographic or filtration methods) of suspected adventitious agents during the processing of human- or animal-derived AM components
3. Testing (e.g., through polymerase chain reaction or virally-responsive cell lines) the initial raw material and final purified human- or animal-derived AM components for the presence of suspected adventitious agents

Industry and regulators have not yet established standard terminology to fully describe the presence or absence of human- or animal-derived components in AMs. Nevertheless, CGT product manufacturers should strive to eliminate all possible risk of adventitious agents in their final CGT products. The most direct way to achieve this goal is to eliminate AMs containing materials of human or animal origin. If AMs containing human- or animal-derived components are used, CGT product manufacturers should require AM suppliers to verify and document the absence of adventitious agents through the proper sourcing, processing, and testing of their human- or animal-derived components.

Regardless of the stated grade of the AM, the CGT product manufacturer is responsible for developing comprehensive and scientifically sound qualification plans to ensure the traceability, consistency, suitability, purity, and safety of the AM. Developers would be well served by referring to the International Council for Harmonisation (ICH)Q8(R2) guideline on *Pharmaceutical Development*, which provides internationally harmonized methodology for incorporating a quality-by-design (QbD) approach into development processes. This guidance allows systematic consideration of the critical quality attributes of a manufacturing process, including materials, resulting in the design of a high quality product.▲ (USP 1-May-2020)

Change to read:

QUALIFICATION OF ANCILLARY MATERIALS

▲AM qualification is the process of acquiring and evaluating data to establish the source, identity, purity, safety, and overall suitability of a specific AM used in the manufacturing process. CGT product developers and manufacturers are responsible for establishing and periodically reevaluating rational and scientifically sound AM qualification programs. These programs should include the characterization and quantification of each AM throughout the manufacturing process (i.e., measuring its tendency to be removed, degraded, or taken up by cells during manufacturing). Although the heterogeneity of many CGT products and AMs makes it difficult to recommend specific tests or protocols for a qualification program, the following provides a practical approach applicable to most AMs.

Thorough documentation is the cornerstone of any qualification program. A well-designed and well-documented AM qualification program becomes more comprehensive as product development progresses. Development of progressive specifications for AMs typically runs in parallel to the development of progressive specifications for the CGT product, including analytical testing. In early stages of CGT product development, safety is the primary focus; a complete description of AM characteristics, and precise quantitation of residual AM in the CGT product, may be limited by a lack of validated assays. In later CGT product stages, AM quality and residuals specifications, along with analytical test methods, should be more comprehensively elaborated to support eventual licensure of the CGT product.

In cases where AMs are products licensed for therapeutic use and manufactured under current good manufacturing practices (cGMP), the extent of qualification is typically less than that for a material intended for research purposes. While use of licensed therapeutic products as AMs may ensure a higher level of quality and safety, it is outside the scope of the licensed product's intended use and labeling; therefore, the AM's suitability for use in the CGT product manufacturing process still requires qualification. Modification of the licensed product, such as dilution, reformulation, mixture with another agent, aliquotting, or repackaging, could trigger additional qualification. Modification of the licensed product's container–closure system may also be necessary if it will be used as an AM.

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In many situations, complex or unique substances essential for CGT product process control or production will not be available in pharmaceutical grade. Even if an AM manufactured in compliance with cGMP is used, the CGT product manufacturer must develop a scientifically sound strategy for AM qualification based on assessments of criticality and principles of risk management. This assessment takes into account major risks to the CGT product, AM or CGT product failure modes, and impacts to the product or patient in the event of an AM or CGT product failure. A qualification program for AMs used in CGT product manufacturing should address each of the following areas: 1) identification and sourcing, 2) selection and suitability for use in manufacturing, 3) characterization, 4) vendor qualification, and 5) quality assurance and control. ▲ (USP 1-May-2020)

Identification ▲and Sourcing▲ (USP 1-May-2020)

The first step in any qualification program is the listing of all of the AMs used in a given ▲CGT▲ (USP 1-May-2020) product manufacturing ▲process, including details on▲ (USP 1-May-2020) where in the manufacturing process they are ▲▲ (USP 1-May-2020) employed. The source and intended use for each material should be established, and the necessary quantity or concentration of each material should be determined. Alternate sources for each material should be identified ▲and qualified.▲ (USP 1-May-2020)

Selection and Suitability for Use

Developers of CGT products should establish and document selection criteria for AMs and qualification criteria for each vendor early in the design phase of ▲CGT▲ (USP 1-May-2020) product development. Selection criteria should include assessments of microbiological and chemical purity, identity, and biological activity pertinent to the specific ▲CGT product▲ (USP 1-May-2020) manufacturing process. It is important to address these issues early in product development because certain AMs that are initially considered necessary may be impossible or prohibitively expensive to qualify, thereby justifying the investigation of alternatives or replacements. Examples include some animal- or human-derived materials that ▲may▲ (USP 1-May-2020) have alternate (i.e., plant or chemically synthesized) sources.

AMs ▲containing components▲ (USP 1-May-2020) of animal or human origin ▲require careful selection because of the▲ (USP 1-May-2020) potential ▲risk of▲ (USP 1-May-2020) infectious or zoonotic disease, ▲and the associated regulatory scrutiny. Vendors should provide documentation on the country of origin for animal-derived AMs to address concerns regarding transmissible spongiform encephalopathies and other diseases of agricultural concern (e.g., tuberculosis, brucellosis, etc.).▲ (USP 1-May-2020) In many cases, the chain of custody for animal-derived AMs (i.e., abattoir → intermediate processing center → final processing center) ▲must▲ (USP 1-May-2020) be documented. Vendors of human-derived AMs should ▲▲ (USP 1-May-2020) supply documentation regarding material traceability. For instance, human plasma-derived AMs should be sourced from licensed facilities that control the donor pool and appropriately screen the individual donors for relevant human infectious diseases. ▲▲ (USP 1-May-2020) Vendors of animal- and human-derived AMs ▲may supply different grades of materials, some more suitable for use in CGT product manufacturing than others.▲ (USP 1-May-2020) Many animal- and human plasma-derived components are subjected to ▲validated▲ (USP 1-May-2020) chemical (detergent or solvent treatment) or physical (heat, ▲irradiation, or filtration)▲ (USP 1-May-2020) treatments that have been shown ▲▲ (USP 1-May-2020) to significantly reduce the risk of ▲contamination by▲ (USP 1-May-2020) adventitious microbial or viral ▲agents.▲ (USP 1-May-2020)

Characterization

▲Quality control and characterization tests for each AM need to be developed (or adopted) and implemented. AM testing should assess a variety of quality attributes, including identity, purity, functionality, and freedom from microbial or viral contamination. The appropriate level of testing for each AM is derived from its risk assessment profile and the knowledge gained during CGT product development. AM test specifications should be established and justified to ensure consistency and performance in the CGT product manufacturing process. Acceptance criteria can be based on data from lots used in preclinical and early clinical studies, lots used for demonstration of manufacturing consistency, and relevant development data, such as those arising from analytical method development and stability studies. AM testing data should be tracked over time for consistency and retained samples should be archived for later analysis, if necessary.▲ (USP 1-May-2020)

Some AMs that are biological in nature may be difficult to fully characterize. Because these materials exert their effects through complex biological activities, and ▲because physicochemical▲ (USP 1-May-2020) testing may not be predictive of the AM's process performance, ▲it is frequently necessary to include▲ (USP 1-May-2020) functional or performance testing. ▲▲ (USP 1-May-2020) Performance variability of ▲AMs▲ (USP 1-May-2020) may have a detrimental impact on the potency and consistency of the ▲CGT▲ (USP 1-May-2020) product. Examples of complex ▲functional▲ (USP 1-May-2020) testing for AMs include growth promotion testing ▲▲ (USP 1-May-2020) of fetal bovine serum (FBS) ▲or human plasma/serum supplements,▲ (USP 1-May-2020) performance testing of digestive enzyme preparations, and in vitro tissue culture cytotoxicity assays (see *Performance Testing*).

Vendor Qualification

Vendors ▲▲ (USP 1-May-2020) of AM should be qualified at the earliest opportunity. Auditing the vendor's manufacturing facility, including their GMP ▲capabilities▲ (USP 1-May-2020) and AM testing program, are basic elements of a vendor qualification program. A review of the vendor's processing procedures and documentation program is essential in establishing confidence in the vendor as a reliable supplier. Vendors certified through an ISO inspection program or audited by ▲▲ (USP 1-May-2020) governmental agencies ▲typically▲ (USP 1-May-2020) have robust quality systems in place. Reports of past audits of US suppliers obtained through the Freedom of Information (FOI) Act may augment the qualification process.

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▲The CGT product developer should establish a written quality agreement with each AM vendor in order to clearly define the roles and responsibilities, including communication channels, change control, audit procedure, and dispute resolution.▲ (USP 1-May-2020) It is important to develop a good working relationship with vendors. In some cases, the vendor may provide higher manufacturing standards, custom formulation services, or replacement of substandard components upon request, with or without additional costs. ▲▲ (USP 1-May-2020) Vendors should be familiar with the principles of validation, especially cleaning, viral inactivation, and sterilization validation. It is also critical to ensure that the ▲AM▲ (USP 1-May-2020) vendor takes appropriate steps to prevent cross contamination between ▲different AMs▲ (USP 1-May-2020) during manufacture. Finally, ▲AM vendors should▲ (USP 1-May-2020) supply written certification of processing or sourcing changes to customers, well in advance of the implementation of the changes so that customers can evaluate the potential impact of such changes.

Quality Control and Quality Assurance

Because the components of the ▲AM▲ (USP 1-May-2020) qualification program are multifaceted and need to be in compliance with cGMP, they should be monitored by a quality assurance/quality control unit (QAU). Typical QAU activities include the following systems or programs: 1) incoming receipt, segregation, inspection, and release of materials prior to use in manufacturing, 2) vendor auditing and certification, 3) certificate of analysis verification testing, 4) formal procedures and policies for out-of-specification materials, 5) stability testing, and 6) archival sample storage.

Change to read:

RISK ▲MANAGEMENT

The evaluation of AM risk to CGT product quality should be based on scientific knowledge, with the ultimate goal of patient protection. ICH Q9 (step 4) "Quality Risk Management" provides a useful approach to risk management principles, process, methods, and definitions. Risk management processes include identifying, analyzing, and evaluating risks, followed by controlling potential risks. Tools or methods, such as a risk evaluation matrix (REM), can quantify risk and facilitate appropriate decision-making and risk acceptance.

Each AM is potentially subject to the risk management process, from selection and qualification to storage and distribution, and use in manufacturing. For reasons described earlier in this chapter, and especially because of the inability to thoroughly remove some AM residuals from the final CGT product, AM selection should incorporate a risk-based approach. Before conducting an AM risk assessment the CGT product manufacturing process must be understood, including identification of the AM's relationship to the CGT product's critical quality attributes (CQAs); these considerations are described in ICH Q11 (step 4) "Development and Manufacture of Drug Substances". Assessment tools can then be used to quantitate each critical parameter, resulting in an assessment of overall risk. For example, if an AM had a suitable safety profile, was used in minimal amounts in upstream steps of the CGT product manufacturing process, and was thoroughly removed from the final CGT product, it would earn a low risk category since both the likelihood of failure (toxicity) and the occurrence severity (consequence to the patient) are low. Conversely, an AM with known toxicity which is introduced downstream would have higher potential to appear as a toxic residual in the final product, and would therefore be assigned to a medium or high risk category. Regardless of the risk category, assessment of AM removal should be completed during CGT product manufacturing process characterization and validation.

As part of the risk assessment process a rational and scientific qualification program should be designed for each AM, taking into account sources and manufacturing processes. Whenever available, AMs that are approved or licensed therapeutic products are preferable because they are well-characterized, possess an established toxicological profile, and are manufactured according to controlled and documented procedures. Incorporating licensed or approved biologics, small molecule drugs, and medical devices or implantable materials into CGT product manufacturing processes presents a more favorable patient safety profile, compared to unapproved versions. Qualification programs for these AMs should reflect the extensive scrutiny to which they were subjected during development and manufacture. Consequently, greater emphasis may then be placed on the impact of inherent AM variability on final CGT product function. For example, if a manufacturer selects approved human serum albumin (HSA) as a cell culture medium supplement for CGT product manufacturing, it may be prudent to assess the impact of the HSA's lot-to-lot variability on cell growth rate and differentiation, its stability during manufacturing, or its interaction with other processing components. Such qualification approaches focus on the AM's variability as it influences final product potency and safety. AM qualification programs should be comprehensive to minimize risks to the consumer and the CGT product.

To aid CGT manufacturers and developers in the design of their AM risk management and qualification programs, potential risk categories are presented as Tiers 1–4 in *Table 1*, *Table 2*, *Table 3*, and *Table 4*, respectively; these are provided as a guide to create an REM. The REM should also consider the amount of the AM used, the stage at which the AM is used in the manufacturing process, and the residual AM remaining in the final CGT product.▲ (USP 1-May-2020)

Tier 1

▲The AM is a highly qualified material that is well-suited for use in manufacturing of CGT products, such as a licensed biologic, drug, or medical device. Tier 1 AMs generally come with sterile packaging systems or dosage forms labeled with their intended use, and the CGT product developer clearly defines and documents their use within the manufacturing process. Changes to the packaging, dosage form, formulation, or storage conditions must be qualified and documented; these changes may also trigger further AM release testing and stability studies.▲ (USP 1-May-2020)

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Tier 2

▲The AM is a well-characterized material produced under an established quality system well-suited for CGT product manufacturing, but the AM is not a licensed or approved medical product. Many Tier 2 AMs are produced specifically for the manufacture of CGT products. Most animal-derived materials are excluded from this category.▲ (USP 1-May-2020)

Tier 3

▲The AM is either intended for research use, locally produced under laboratory conditions, or not intended for use in CGT product manufacturing; it may be approved by regulatory agencies as part of an in vitro diagnostic device. Tier 3 AMs require more qualification than Tier 1 or Tier 2 materials. Using a risk-based approach, an AM manufacturing process upgrade may be necessary to fully qualify the AM for CGT product manufacturing. For example, robust viral inactivation and removal steps may need to be added to the production process for a diagnostic-grade monoclonal antibody in order to produce a reagent suitable for CGT product manufacturing. The manufacturing process for an AM produced in an academic or pilot-stage manufacturing facility will likely need to be upgraded for late-stage clinical trials and eventual commercialization of the CGT product.▲ (USP 1-May-2020)

Tier 4

▲The AM is produced for industrial or research use and may contain harmful impurities, and/or may contain animal- or human-derived components harboring adventitious agents. High-risk Tier 4 AMs require extensive qualification before they may be used in CGT product manufacturing. These materials may require one or more of the following measures:

1. An upgrade of the AM manufacturing process
2. Selection, screening, and testing of the animal or human sources
3. Testing of each AM lot to ensure that it is free of adventitious agents or specific contaminants
4. Treatment of the AM to inactivate or remove adventitious agents or specific contaminants
5. Validation of the CGT product manufacturing process to assess consistency of removal of a known toxic substance, and/or lot-release testing to demonstrate safe residual levels
6. Validation of the CGT product manufacturing process to demonstrate removal or inactivation of adventitious agents or specific contaminants

For Tier 4 AMs deemed critical to the CGT product manufacturing process, CGT product developers should explore alternative AMs or sources as early as possible in development.▲ (USP 1-May-2020)

Table 1. AM Risk Tier 1 ▲Materials Intended for Use as Approved Biologics, Drugs, or Medical Devices▲ (USP 1-May-2020)

Example	Typical Use in CGT Product Manufacturing	Qualification or Risk Reduction ▲▲ (USP 1-May-2020)
Recombinant insulin for injection	▲Chemically defined basal cell culture medium▲ (USP 1-May-2020)	• DMF cross reference (when possible or practical) • Certificate of analysis • Assess lot-to-lot effect on process performance[b] • Assess removal from final product • ▲Assess AM stability as stored and used in CGT product manufacturing[c] • Visual inspections • Assess particulates and extractables▲ (USP 1-May-2020)
Organ ▲transport/▲ (USP 1-May-2020) preservation fluid	Process biological fluid employed in tissue transport or processing	
Human serum albumin for injection	Cell culture ▲additive▲ (USP 1-May-2020)	
Sterile fluids for injection	Process biological fluid employed in tissue transport, cell processing, purification	
Implantable ▲materials▲ (USP 1-May-2020) (collagen, silicone, polyurethane constructs intended for surgical implantation)	Scaffolds, matrices for immobilized cellular cultivation	
Recombinant deoxyribonuclease for inhalation or injection	Process enzyme employed in viral vector manufacturing, stem cell processing	
Injectable antibiotics[a]	▲▲ (USP 1-May-2020) Biopsy transport fluid additive to reduce risk of bacterial contamination	
Injectable monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Injectable cytokines, ▲vitamins, chemicals, nutrients▲ (USP 1-May-2020)	Cell culture ▲additive▲ (USP 1-May-2020)	
▲▲ (USP 1-May-2020)	▲▲ (USP 1-May-2020)	
IV bags, transfer sets and tubing, cryopreservation bags, syringes, needles	Storage vessels or container–closure systems, closed aseptic transfer systems	

[a] Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity.
[b] Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity. See *Performance Testing*.
[c] Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrate the stability and preservation of activity of the AM under the conditions that are specific to the manufacturing application.

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Table 2. AM Risk Tier 2 ▲Well-Characterized Materials with Intended Use as AMs▲ (USP 1-May-2020)

Example	Typical Use in CGT Product Manufacturing	Qualification or Risk Reduction ▲ (USP 1-May-2020)
Recombinant growth factors, cytokines[a]	Cell culture ▲ (USP 1-May-2020) additive	▲All of the qualification and risk mitigation activities in Tier 1, plus the following:▲ (USP 1-May-2020) • DMF cross reference (when possible or practical) • ▲Confirm certificate of analysis test results that are critical to CGT product identity, purity, or potency • Verify AMs containing animal- or human-derived materials have been purified, tested, and certified to be free of adventitious agents▲ (USP 1-May-2020) • Vendor audit
Immunomagnetic beads	▲Cell selection▲ (USP 1-May-2020)	
Human AB serum	Cell culture ▲ (USP 1-May-2020) additive	
Progesterone, estrogen, vitamins, purified chemicals (USP-grade)	Cell culture ▲ (USP 1-May-2020) additives, induction agents, buffer components	
Sterile process buffers	Process biological fluid employed in tissue transport, cell processing, purification	
Biocompatible polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Proteolytic enzymes	▲Digest tissue or protein▲ (USP 1-May-2020)	
Tissue culture media	Cell culture ▲ (USP 1-May-2020) additive	
▲ (USP 1-May-2020)	▲ (USP 1-May-2020)	
Density gradient media	Cell separation via centrifugation	

▲[a] AMs▲ (USP 1-May-2020) produced from nonmammalian, recombinant sources (i.e., microbially grown in the absence of animal-derived growth medium components).
▲ (USP 1-May-2020)

Table 3. AM Risk Tier 3 Moderate-Risk Materials Not Intended for Use as AMs▲ (USP 1-May-2020)

Example	Typical Use in CGT Product Manufacturing	Qualification or Risk Reduction ▲ (USP 1-May-2020)
Recombinant growth factors, cytokines	Cell culture ▲ (USP 1-May-2020) additive	▲All of the qualification and risk mitigation activities in Tier 2, plus the following:▲ (USP 1-May-2020) • DMF cross reference (when possible or practical) • ▲Confirm certificate of analysis test results▲ (USP 1-May-2020) • Upgrade manufacturing process ▲and/or testing▲ (USP 1-May-2020) for material to ▲be suitable for therapeutic use▲ (USP 1-May-2020)
Tissue culture media	Cell culture ▲ (USP 1-May-2020) additive	
Monoclonal antibodies ▲(for diagnostic use▲ (USP 1-May-2020) produced in cell culture)	▲Targeting cells for selection, activation, or removal▲ (USP 1-May-2020)	
Process buffers	Process biological fluid employed in tissue transport, cell processing, purification	
Novel polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Proteolytic enzymes	▲Digest tissue or protein▲ (USP 1-May-2020)	
Purified chemicals (reagent-grade)	Culture medium additives, induction agents, buffer components	

▲ (USP 1-May-2020)

Table 4. AM Risk Tier 4 High-Risk Materials

Example	Typical Use in CGT Product Manufacturing	Qualification or Risk Reduction
▲Animal- and human-derived materials▲ (USP 1-May-2020)	Cell culture ▲ (USP 1-May-2020) additives; ▲targeting moieties for cell selection, activation, or removal▲ (USP 1-May-2020)	▲All of the qualification and risk mitigation activities in Tier 3, plus the following: • Safety testing for residuals in CGT product • Recombination-competent retroviral testing for relevant gene therapy AMs▲ (USP 1-May-2020) • Adventitious agent testing for animal source-relevant viruses
▲ (USP 1-May-2020)	▲ (USP 1-May-2020)	
Animal-derived polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Purified enzymes	Process ▲additives such as trypsin or collagenase for digesting protein or tissue▲ (USP 1-May-2020)	
▲ (USP 1-May-2020)	▲ (USP 1-May-2020)	
Animal or human cells used as feeder layers	Cell culture substratum or ▲conditioned medium▲ (USP 1-May-2020)	
▲Toxic entities (i.e., methotrexate, bacterial toxins, etc.)	Cell culture additives to maintain transgene expression, enhance proliferation, or improve cell survival upon cryopreservation▲ (USP 1-May-2020)	

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Change to read:

PERFORMANCE TESTING

▲In cases where an AM is chosen for its effect on a particular biological function of an intermediate or final CGT product, performance testing is an essential element of its overall qualification. This is especially true when the AM plays a critical role in modulating a complex biochemical effect or has a large impact on yield, purity, and/or final product potency. Performance testing is particularly critical for AMs that are complex substances, mixtures, or biologically sourced; these AMs are more likely to show significant lot-to-lot variability, lack a simple identity test, and be difficult to characterize. The development of well-defined performance assays for complex AMs will not only ensure process reproducibility and final product quality, but in many cases will satisfy regulatory requirements for identity testing.

Often, the initial qualification of an AM for use in CGT product manufacturing investigates the dose-response effect of the AM on yield, purity, or potency of the therapeutic product. The optimal amount of AM used in CGT product manufacturing should consistently yield the desired effect, while minimizing residuals. Performance testing frequently utilizes a scaled-down or simulated manufacturing process.

Some examples include:

- For a cellular proliferation AM added to culture medium, the assay could demonstrate that each lot of AM produces the expected rate and degree of cellular proliferation
- For a monoclonal antibody used to purify a particular cell type, the assay could demonstrate that each new lot of monoclonal antibody purifies a standard cell population with the expected recovery and purity
- For a density gradient material used to purify a vector or cell, the assay could demonstrate that each new lot of material purifies the vector or cell with the expected recovery and purity
- For a helper viral vector used to produce a gene therapy vector, the assay could demonstrate that new lots of the helper vector produce the expected amounts of active gene therapy vector

Functional assays may evolve as CGT product manufacturing processes develop, and the critical relationships between AM and final products are better understood.

When performance tests yield relative results, it is helpful to compare each new lot of AM side by side with a reference standard AM, if available. This simultaneous comparison reduces the variability due to different lots of cells or vectors used in testing.▲ (USP 1-May-2020)

Change to read:

▲RESIDUAL▲ (USP 1-MAY-2020) ASSESSMENT AND REMOVAL

▲Though AMs are not intended to be present in the final CGT product, it is often challenging to completely remove AMs without impairing the CGT product yield or quality. To establish appropriate specifications for AM residuals, developers should address both AM removal procedures and AM analytical methods early in development. Developing appropriate residuals specifications and detection methods ensures CGT product and process consistency, allows characterization of the final CGT product, and reduces negative effects on product quality, clinical efficacy, and safety.

Thresholds for AM residuals are established, and may evolve, throughout product development. In early development, the fold reduction in AM concentration is often estimated for each manufacturing unit operation, and AM removal is typically expressed as a dilution factor or log reduction factor. In late development, direct residuals measurements utilize validated assays. However, all AM residuals measurements must be interpreted with some caution, because intracellular and extracellular AM concentrations may differ.

As a part of CGT product manufacturing process validation, spiking experiments with high AM concentrations may be required to mimic a worst case scenario and demonstrate manufacturing process robustness. Validation studies should incorporate the following considerations:

1. Residuals assay should accurately quantitate the AM in each sample matrix
2. If a scaled-down validation is conducted, the comparability of the small-scale and full-scale processes must be demonstrated
3. In spiking studies, the higher AM levels must not interfere with the purification process

Specifications for residual AM in the final CGT product are typically based on the residual AM in the CGT product lots used in toxicological or clinical studies, or on known toxicological data.▲ (USP 1-May-2020)

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CONCLUSION

▲AM quality is critical to CGT product quality. AMs must be carefully selected, characterized, and qualified, so that they perform consistently and as intended in the CGT product manufacturing process. AM qualification programs should focus on developing appropriate AM specifications, robust CGT product manufacturing processes, and AM risk assessments and mitigations, in order to prevent negative impacts on CGT product quality, clinical efficacy, and patient safety.▲ (USP 1-May-2020)

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APPENDIX

Lists of Relevant *USP* Chapters and Regulatory References

AMs used in CGT products will be regulated in the context of the manufacturing process of the CGT products. ▲▲ (USP 1-May-2020) The following lists of documents include relevant regulatory ▲and technical guidances on▲ (USP 1-May-2020) product and process development, manufacturing, quality control, and quality assurance.

USP Chapters

- *Biological Reactivity Tests, In Vitro* ⟨87⟩
- *Biological Reactivity Tests, In Vivo* ⟨88⟩
- ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020)
- ▲*Gene Therapy Products* ⟨1047⟩▲ (USP 1-May-2020)
- *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩
- *Capillary Electrophoresis* ⟨1053⟩
- *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩
- *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩
- *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩
- *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩

Code of Federal Regulations (CFR)

▲Regulations	Title
21 CFR 211 Subpart E, 211.80 through 211.94 and 211.101	Control of Components and Drug Product Containers and Closures
21 CFR 312	Investigational New Drug Application
21 CFR 314	Applications for FDA Approval to Market a New Drug
21 CFR 801.109(b)(1)	Labeling—Prescription Devices
21 CFR 807 Subpart E, 807.81 through 807.97	Premarket Notification Procedures
21 CFR 812	Investigational Device Exemptions
21 CFR 814	Premarket Approval of Medical Devices
21 CFR 876.5885	Tissue Culture Media for Human Ex Vivo Tissue and Cell Culture Processing Applications▲ (USP 1-May-2020)

FDA Guidance Documents

- ▲▲ (USP 1-May-2020) *Guidance for Industry: Monoclonal Antibodies Used as Reagents in Drug Manufacturing*, ▲March 2001. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm080417.pdf.▲ (USP 1-May-2020)
- *Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*, ▲May 1993. https://www.fda.gov/downloads/biologicsbloodvaccines/safetyavailability/ucm162863.pdf.▲ (USP 1-May-2020)
- *Class II Special Controls Guidance Document: Tissue Culture Media for Human ex vivo Tissue and Cell Culture Processing Applications—Final Guidance for Industry and FDA Reviewers*, May 16, 2001. ▲https://www.fda.gov/RegulatoryInformation/Guidances/ucm073567.htm.
- *Guidance for Industry: Contract Manufacturing Arrangements for Drugs: Quality Agreements*, November 2016. https://www.fda.gov/downloads/drugs/guidances/ucm353925.pdf.
- *Guidance for Industry and Food and Drug Administration Staff: Use of International Standard ISO 10993-1, "Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process"*, June 16, 2016. https://www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm348890.pdf.▲ (USP 1-May-2020)

National and International Regulatory Documents

- ICH Q5A: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human and Animal Origin. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q5D: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products. ▲Available at: http://www.ich.org.
- ICH Q8(R2): Pharmaceutical Development. Available at: http://www.ich.org.
- ICH Q9: Quality Risk Management. Available at: http://www.ich.org.▲ (USP 1-May-2020)
- Public Health Service Guideline on Infectious Diseases Issues in Xenotransplantation. ▲Available at: https://www.cdc.gov/.
- Health Canada "Guidance Document: Preparation of Clinical Trial Applications for use of Cell Therapy Products in Humans". Available at: https://www.canada.ca/en/health-canada.html.▲ (USP 1-May-2020)

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⟨1044⟩ CRYOPRESERVATION OF CELLS

INTRODUCTION

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability. The purpose of cryopreservation is to bank the cells and allow their future use in in vitro or in vivo applications for which post-thaw function is sufficiently representative of the cells' prefreeze function. Cryopreservation also minimizes the risk of genetic mutation or development of subpopulations due to cell replication. Depending on the application, sufficient postcryopreservation function may be assessed by the ability to divide, proliferate, differentiate, express genes, or to produce proteins, or by another specific functional property.

This chapter presents best practices for cryopreservation, maintenance, and use of a wide range of cells, cell therapy products, and cell banks derived from a variety of sources including human, animal, and microbial cultures (the chapter also contains an *Appendix* with additional guidance documents that are useful for particular cell types and applications). Cryopreserved cells provide a ready source of viable cells that can be used, either directly or indirectly, for the purposes of diagnostic tests, therapy, manufacture of drug products and vaccines, and for bioassays used to evaluate the potency of therapeutic drugs and vaccines. In some cases the cells themselves, after cryopreservation and thaw, constitute the patient therapy, and in other cases the cells are propagated or otherwise manipulated ex vivo in order to generate the product (e.g., a culture-expanded cellular therapy, a therapeutic protein, a monoclonal antibody, or a vaccine). In all cases, proper cryopreservation is essential for retention of required cellular properties and, ultimately, for application toward the advancement of patient therapies.

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PRINCIPLES OF CRYOPRESERVATION

Overview

Understanding the role of water and the need to adequately remove it from cells or abrogate its ability to form ice crystals, which damage the cell membrane, is critical to successful cryopreservation. When cells are frozen in aqueous suspension, often they are destroyed. However, in the 1940s Polge and others discovered the cryoprotective properties of glycerol. Since then several chemicals, generically called *cryoprotectant agents* (CPAs), have been identified. The mechanism of action of CPAs is complex and is not fully understood. However, according to the commonly accepted theory of *colligative* action, CPAs increase solute concentration both within the cell and extracellularly, thereby suppressing ice formation. For this purpose, the so-called penetrating (or intracellular) CPAs [e.g., dimethylsulfoxide (DMSO), glycerol, propanediol, and methanol] must be able to cross the cell membrane readily and penetrate the cell without significant toxicity. There also is a group of nonpenetrating (or extracellular) CPAs (e.g., sucrose and trehalose) whose mechanism of action is thought to be related at least in part to their stabilizing interaction with cell membranes. This property also may explain the cryoprotective activities of certain large molecular weight compounds such as hydroxyethyl starch and polyvinylpropylene. Theoretical models of cryoprotection typically evoke the colligative theory, but full explanation of CPA action is yet to be established.

An alternative form of cell preservation, commonly called *vitrification,* whereby the cell suspension is loaded with high levels of penetrating CPAs (often several in combination), induces a glass-like state in which cellular and extracellular water cannot readily form ice crystals. When cell suspensions prepared in this way then are cooled very rapidly (cooling rates of 100°–1000°/min or more) the extreme viscosity prevents osmosis, and the water molecules are unable to form ice. This procedure has been widely used for complex structures including a variety of human, plant, and animal tissues and may help preserve those cell preparations that have variable degrees of cellular permeability or when standard cryoprotection cannot deliver the range of conditions required to optimally preserve viability in all the tissues' component cell types.

CPAs have biological activities beyond their cryoprotective properties. Some, like DMSO, can affect the cell membrane, cytoskeleton, and gene expression and may be toxic to cells following prolonged exposure. Therefore, during development of new cryopreservation protocols analysts should perform a toxicity assay in which the cells are exposed to the CPA over a range of time intervals to evaluate loss of viability or alteration of functionality.

Key Elements of Cryopreservation Practice

For any cellular sample or therapeutic product being cryopreserved, method development should address the following elements:

PREFREEZE PROCESSING AND CHARACTERIZATION

Optimizing the condition of the cells immediately before cryopreservation is critical to a successful outcome. The nature and extent of prefreeze processing depends on the state of the original cells harvested for preservation, the composition of the cell suspension, and the specific processing steps leading into cryopreservation. Prefreeze processing may include selection of subpopulations, ex vivo expansion, or incubation with activating or priming factors.

Precryopreservation characteristics and identity should be established during early process development. For cell banks in particular, the cell status and optimal growth conditions, as well as documented history (with traceability to a qualified cell bank or acceptable source), characteristics, and authenticity should be documented. Cell status and history typically are described in terms of the nature and number of manipulations and culture passages from the primary cells or original isolate. Finite or primary cells usually are cryopreserved at an early passage to maintain integrity of the original tissue, but continuous

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cell lines may be cloned and expanded, ensuring a homogeneous cell population. It is recommended to prepare cell banks from a single preparation or expanded population of cells since it is often necessary to pool cells for freezing from multiple culture vessels. Cells from cultures with different passage histories and certainly from different donors should not be pooled. In both cases, analysts should maintain detailed records of the procedures.

To prepare for cryopreservation of cultured cells, cells should be harvested during exponential or the most rapid phase of growth and before the culture enters stationary phase. Harvesting cells during this phase ensures that the cells are most viable and uniform. The optimal concentration of cells will depend on the cell type, purpose, and best recovery. Typically this lies between 10^6 and 10^7/mL for manufacturing cell banks but may be different for other purposes. Complete growth medium renewal a day before cell harvest also can be beneficial. Additionally, most cell suspensions benefit from washing by centrifugation and resuspension in an isotonic medium to a specific cell concentration. Prefreeze processing should not result in cells that are stressed before the start of the freezing process, or cell losses during freezing or after thaw will be higher than expected.

Optimizing the growth conditions of a cell line or primary cells is important to maintain high viability of the cells in culture. Typically, cells growing actively and in exponential phase have a low cytoplasm to nuclear volume ratio, which is conducive to successful cryopreservation with penetrating cryoprotectants. Suboptimal or improper culture conditions may result in lower viability and cell states that will be less robust for preservation and recovery. The culture medium should be optimized and the same medium should be used throughout all experiments, and each batch of animal-derived materials (e.g., serum) and other culture reagents should be qualified (e.g., see the 2010 WHO guidance and the FDA 2010 guidance referenced in the *Appendix*). If possible, it is recommended to not use animal-derived components in the culture medium particularly for cells used for therapy or as manufacturing substrates.

Per the WHO 2010 guidance and based on a risk assessment, either the Master Cell Bank (MCB) or the Working Cell Bank (WCB) must be tested for adventitious agents. Ideally, samples of cells should be tested for adventitious agents before freezing. The specific testing regimen for potential microbial or viral contamination of cells depends on the donor source, the culture history, and the intended use. Detailed records of the cell history should be maintained as a basis for appropriate risk assessment to direct supplementary testing that may be required (e.g., exposure to bovine viruses in bovine serum albumin). Specific regulatory requirements for testing of cells, or donors of the cells, for products intended for a particular use (e.g., cell therapy or vaccine manufacture) are based on past experience regarding key agents that must be included or considered. *USP* general chapter ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020) contains guidance about sterility and safety testing requirements for cell therapy products.

REAGENTS AND CONTAINERS

All cryoprotectants, containers, etc., should be fit for purpose as indicated in relevant regulatory guidances. Sterile, single-use, disposable plastic bags, cryovials, or straws are customarily used for cryopreservation. Manufacturers' specifications should be carefully reviewed to ensure that the material used to manufacture the cryocontainer is appropriate for use at the storage temperature, is chemically compatible with the contents, minimizes the potential for leachables and extractables, and assures container closure integrity. If straws are used, then primary or secondary containment during storage is important to prevent direct contact of the preserved cells with liquid nitrogen. Cryovials should be selected based on their ability to provide adequate cell bank integrity.

Preservation of cells typically requires the use of specialized solutions that contain a base (typically an isotonic saline-based solution) with CPAs (most commonly DMSO but sometimes glycerol) and sometimes proteins (fetal bovine serum, human serum or plasma, conditioned medium, or human albumin). The optimum composition for different cell types may need to be determined.

The types of vials, labels, ink, or markers used should withstand extreme liquid nitrogen temperatures. The markings on the label should be legible and barcoded if possible. The minimum information on the label should include name or description of cell population, date of cryopreservation, lot number, and passage number if needed. Since most cryolabels are very small, additional information can be included on associated documentation. In certain applications it may also be necessary to sequentially number vials within a single lot as part of the minimum information on the label, to enable better control over movement of vials from a single bank, and to identify sectors of the bank which may have received different cryopreservation conditions.

ADDITION OF CRYOPROTECTANT SOLUTION

Cryoprotectant solutions typically are hypertonic and are not physiological. For example, a 10% DMSO solution used commonly in cell preservation has a concentration of approximately 1.4 osmolarity (Osm/L). Cells introduced into this type of solution rapidly dehydrate as water leaves the cell in order to reduce the difference in osmotic potential between the inside and outside of the cell. DMSO slowly permeates the cell to re-equilibrate. This may cause excursions in volume that can result in a loss of cell viability. Therefore, cryopreservation solutions commonly are added to a cell suspension in stepwise additions or gradually (e.g., using a syringe pump) or slowly dispensing down the side of the container to prevent cell losses resulting from osmotic stress. The method for introducing or removing a cryopreservation solution should be developed and evaluated for its impact on cell viability and functionality.

In the case of DMSO, a large latent enthalpy of mixing results in sample heating when the two solutions are mixed. This heating can be high enough to damage the cells, so solutions that contain DMSO commonly are precooled before mixing. Prechilling the solution reduces heating associated with mixing of the solution, reduces the osmotic volume changes that the cells experience, and reduces cell losses associated with exposure to DMSO. The time that cells are exposed to the cryoprotectant, prior to freezing, should be limited and the maximum time allowable, without deleterious effects, should be determined during development work for routine use.

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COOLING

Two different types of freezing typically are used for cells: controlled-rate cooling (using programmable freezers) and passive cooling (including use of insulated containers). Controlled-rate freezers are attached to liquid nitrogen supplies. The temperature of the chamber should be controlled by increasing or decreasing the flow of cold nitrogen gas into the chamber according to a preprogrammed step. Controlled-rate freezing protocols typically involve several steps, each of which should be evaluated and qualified for a specific cell type.

The use of controlled-rate freezing provides more precise control of the freezing environment and therefore may provide more consistent (and higher) post-thaw recovery for cells that may have a narrow range of cooling rates associated with maximum survival or cells that are sensitive to the temperature at which ice forms in the extracellular solution. Temperature probes placed near the cells being frozen, or in a mock cell suspension that undergoes cryopreservation simultaneously, are used to monitor the freezing process and to provide process control. If release of the latent heat of fusion is delayed or poorly controlled, cells undergoing cryopreservation may be damaged and may have diminished viability after thaw.

Disruption of the controlled-rate freezing during the protocol may occur and typically is caused by failure of a valve in the controlled-rate freezer or cryogen. Protocols for handling disruption of the freezing process and backup plans should be prepared.

Passive freezing involves placing a product in a freezer (about –80° or –150°) and permitting the sample to cool in an uncontrolled fashion. Insulation or specially designed boxes are used to slow the cooling rate for the sample. The average cooling rate achieved for the majority of the process and the consistency of freezing curves should be evaluated and qualified for purpose. In general, control of the thermal environment during freezing results in improved post-thaw recovery, but certain cells exhibit comparable post-thaw recovery when they are passively cooled.

CRYOGENIC STORAGE, SAFETY, AND TRANSPORT

After the freezing process has been completed, products are transferred from controlled-rate or mechanical freezers to cryogenic storage units. Some microbial cell cultures can be suitably maintained in mechanical freezers but this should be demonstrated. Sample warming should be minimized during transfer of the cell product from the freezing device to storage. Cold tables or insulated transfer devices can be used to minimize warming during transfer. Newly cryopreserved cells commonly are placed in a quarantine cryogenic storage unit before completion of testing for adventitious agents. After testing, cells that test negative for adventitious agents can be released for transfer into long-term cryogenic storage units.

The inventory system (or repository) for the maintenance of the cryopreserved cells should be designed for easy access to minimize specimen handling, and the number of times per day that a repository is accessed should be limited because exposure to warmer temperatures may compromise cell viability and, consequently, longer-term stability. Cell banks (e.g., MCBs) or other cell cultures that are accessed infrequently should be stored separately from WCBs or other cell cultures that are accessed more often. Frequent retrieval from the cell bank/culture may cause shifts in temperature. This activity must not compromise the long-term stability and performance of the infrequently used cell bank/culture. It is also valuable to divide a bank and store it in multiple locations to decrease risks due to a catastrophic event at a particular site.

When storing cryopreserved cells, analysts should ensure that the storage temperature does not rise above a critical temperature called the glass transition temperature. For long-term storage of fastidious specimens such as cell lines and primary cell cultures, this critical temperature is not warmer than –130° for nonclinical specimens or not warmer than –150° for clinical material (to give an adequate margin of error) in the vapor phase of the liquid-nitrogen freezer. Liquid-nitrogen freezers are prone to temperature gradients in the vapor phase based on the shape and design of the freezer and the level of liquid nitrogen. Although storing cryopreserved cells in liquid nitrogen prolongs longevity, hazards associated with unsuitable containers or container use (e.g., exploding vials and rupturing bags) have prompted greater use of nitrogen vapor phase storage. Liquid-nitrogen vapor phase provides a more convenient and safe environment for vial retrieval. If the liquid-nitrogen freezer is suitably configured, the working temperature in the vapor phase is commonly –150° or colder. The liquid-nitrogen freezer should be qualified, and the temperature of the vapor phase should be routinely checked to ensure that the temperature does not become warmer than –130° for cell lines or other frozen material or warmer than –150° for material used for clinical applications (e.g., cell therapies).

Temperature-monitoring systems should permit recording and storage of temperature history for quality control purposes. Storage units should be attached to alarms and facility monitoring systems. Critical storage units should be equipped with a multilevel alarm system to ensure backup in the monitoring and response. The storage units should be routinely monitored for temperature failure caused by power disruption and any other potential malfunctions. In the event of equipment or power failure, backup refrigeration should be available.

Proper operation of a repository requires monitoring of temperature and liquid-nitrogen levels and automatic filling. In addition, it is recommended to have a backup for emergency cooling (e.g., empty backup cryogenic storage) in case of freezer failure.

Only individuals who are trained for this purpose should access cryopreserved products or samples. In some cases, verification by a second person is required for source traceability. Personnel assigned to the implementation of the protocols should be trained in standard operating procedures (SOPs). Sample tracking systems that incorporate computer software and sometimes barcoding for identification, logging, and tracking of frozen samples are particularly useful for large sample repositories and may facilitate rapid retrieval of samples and minimize time that the entire repository is exposed to the risk of temperature excursions. In addition, it is recommended that all changes to cryostorage inventories be recorded in log books near the storage unit.

Products and samples such as primary cells, cell lines, and cell therapy products routinely are shipped among sites of collection, processing, storage, and use. Cryopreserved cells typically are shipped in liquid-nitrogen vapor shippers with temperature-monitoring systems to ensure that the unit does not become warmer than –130° for cell lines and –150° for clinical material during the shipping process. Shipping containers are subjected to significant vibrations and mechanical stresses during

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shipment and should be evaluated on a regular basis for proper function. Shipping validation studies should cover worst case scenarios and include temperature monitors for critical materials.

Cryopreserved cells, whether shipped nationally or internationally, should be transported using local postal, US Department of Transportation, and International Air Transport Association guidelines. Packages also should meet other regulatory requirements for quarantine, biosafety, and biosecurity. Cryopreserved cells should be retrieved, packed, and shipped in a manner that does not interfere with the integrity of the cells. For most cryopreserved cells, shipping in dry ice for short duration may be adequate, but the shipping process should be validated, shown to have no adverse impact on the cells, and temperature monitors should be included. However, some cells may require shipping in liquid-nitrogen vapor phase (Dewars). Prevalidation of the shipping methods may be required to determine the best option and prevalidation risk assessment should be performed even if only one option for transport is being considered. With the shipment, shippers should include instructions for proper storage upon cell receipt.

THAWING

Cells frozen using conventional methods (controlled rate or passive freezing) or by vitrification should be thawed as rapidly as possible, and the thawing process starts as soon as the frozen sample is removed from storage. Slow warming rates result in recrystallization damage or exposure of the cells to high extracellular concentrations of CPAs, either of which can result in cell death. For each cell therapy product and cell line the most appropriate thawing procedure (temperature, gradient, and time) needs to be developed. These products and cell lines typically are thawed in a warm-water bath or for therapeutic cell preparations in a bead bath or thermoblock. The water bath should be cleaned regularly and should contain sterile water or *Water for Injection*. The temperature of the bath also should be monitored. Many clinical labs use plastic overwrap bags to hold the primary container during rapid thaw to reduce the risk of product contamination in case the integrity of the inner container is impaired. Alternatively, warm bead baths (usually approximately 37°) can be used to reduce contamination risks. Thawing rates should be as rapid as possible (>1°/s for most mammalian cells). Increasing bath temperatures above 42° to increase the warming rate must be done with extreme caution because hyperthermic temperatures can damage cells, inducing necrosis or apoptosis.

POST-THAW PROCESSING AND EVALUATION

Because cryopreservation solutions are not physiological, it is not uncommon for some post-thaw processing to be performed. For cells preserved in DMSO, cells typically are washed or diluted immediately post-thaw because this CPA is harmful in particular to frozen and thawed cells. Cells are more sensitive to expansion than contraction, so CPA removal or dilution protocols must be carefully optimized to prevent cell losses from dilution or removal.

Quantifying the viability of cells post-thaw is important and may be performed by a variety of methods depending on the post-thaw requirements of the cells. Minimum viability limits should be set based on experience and thawed products with viabilities below the set limits should be discarded. The process of cryopreservation subjects the cells to significant stresses that can alter metabolic function, membrane structure, etc. Therefore, development and validation of suitable post-thaw assays are critical. Post-thaw function is most commonly assessed using physical integrity (e.g., membrane integrity), metabolic activity, mechanical activity (attachment or contraction), mitotic activity, or engraftment potential. The selection of assay depends strongly on the desired post-thaw function of the cell. Membrane integrity is used most often. Today, dyes like trypan blue are used less often to measure post-thaw physical integrity because the dye is difficult to validate on frozen and thawed cells. The method to test viability needs to be carefully selected and qualified for the particular cell type being measured with a protocol that specifies diluents and time. Fluorescent dyes are used with increasing frequency to determine cells' post-thaw physical integrity. Rigorous methods of post-thaw assessment typically involve multiple measures of cell viability and, in particular, at least two independent assays to measure post-thaw viability. For example, post-thaw attachment and proliferation commonly are used to evaluate viability. As the complexity of the desired cell function after thawing increases, so do the demands on post-thaw assessment. For example, post-thaw assessment of stem cells may require assay of membrane integrity as well as proliferation and the ability of the cells to differentiate into different lineages post-thaw. Post-thaw assays should be carefully developed and validated to avoid measurement bias. A certain fraction of cells will lyse during freezing, and methods of measuring cell recovery should include a complete assessment of cell losses (cells that have lysed as well as cells that are intact but not viable). Stability of cryopreserved cells can be assured by periodically thawing and testing a vial of the cells (also see ICH Q5D).

Testing for adventitious agents after the preparation of MCBs and WCBs should be routine, and *USP* general chapters *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and *Virology Test Methods* ⟨1237⟩ provide additional testing guidance (see also the FDA 2010 guidance cited in the *Appendix*). Testing for mycobacterial contamination, which may not be isolated in standard sterility testing, also can be considered for some cell substrates. Representative vials should be retrieved and tested for contamination (bacteria, fungi, *Mycoplasma,* and viruses). Numerous well-established methods are available for detecting *Mycoplasma* in cell cultures (see *USP* general chapter *Mycoplasma Tests* ⟨63⟩).

The batch record should be detailed, including the history of the cells and all activities starting from their receipt to release of the cell banks or products for use. The record should include detailed information about the cryopreservation process, including the procedure, the equipment used (with unique identifier), and a printed record of the freeze profile. The viabilities of the cryopreserved cells should be monitored over time to ascertain the effectiveness of the freezing process and the storage conditions. The information captured in each batch file of a cell line must be traceable to the original source, and all documents should be maintained and updated according to the quality management system in place.

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CRYOPRESERVATION OF HUMAN CELL THERAPY PRODUCTS

Cell preservation methods are used to ensure product stability during hold, storage, and transport steps for a wide range of human cell-based products (additional information is found in ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020)). Cells can be preserved in liquid suspension for up to a few days, but quantitative and qualitative changes in the cellular product invariably occur over time. Although preservation in the frozen state also affects the cellular product, it allows more predictable preservation of specific product characteristics over much longer time intervals.

For any given cell product, the decision to use cryopreservation depends primarily on the timing of final product administration in relationship to cell source collection and product manufacturing steps. Many patient-specific autologous and allogeneic products are maintained in liquid suspension, without cryopreservation, from starting cell source through final formulation and are released as fresh products after a relatively short time. However, many clinical applications require cryopreservation of the cell source, intermediate products, or final product. In these cases, cryopreservation can permit optimization of workflow during manufacturing, completion of lot-release testing, maintenance and management of a product inventory, transport of the product to the clinical site, and coordination of product administration with the patient's medical or surgical regimen. For example, umbilical cord blood is cryopreserved and stored in public banks for subsequent transport to, and temporary storage at, clinical transplant centers, where it is thawed immediately before infusion into a patient.

Development of a cryopreservation process for a clinical cell therapy must consider the consequences for the product, the patient, and the overall feasibility of the therapy. For the cell product, the manufacturer must ensure that expected cell losses because of cryopreservation and thaw occur in a manner that is reasonably predictable and must ensure that the final product administered to the patient will meet specifications for cell number, viability, and functional characteristics.

For the patient, cryopreservation may affect the efficacy and safety of the final product. For example, the cryoprotectant DMSO is associated with risk of predictable dose-dependent gastrointestinal, cardiovascular, and neurologic toxicities that typically are ascribed to histamine release. Residual DMSO in the final product should be estimated or measured. DMSO is categorized by ICH as a Class 3 (relatively low risk) solvent or excipient in pharmaceutical products, and amounts of up to 50 mg/day, or less, are considered acceptable without justification (see ICH Q3C). Cryopreserved cellular products frequently contain 10–20 times this amount unless they are washed after thawing. Even higher amounts of DMSO may occur with administration of multiple cryopreserved products, and this occurs commonly with autologous peripheral blood stem cell transplantation. A DMSO limit of 1 g/kg recipient weight/day is commonly used in clinical cell therapy practice. Procedures to prevent DMSO toxicity also should be considered. It is common clinical practice to premedicate patients with diphenhydramine or other antihistamine agents to prevent DMSO toxicity. Product washing methods by either manual (centrifugation) or automated methods also can be considered but must be validated to ensure adequate postwash recovery and cell function.

Use of cryopreserved cell therapy products may require the clinical site to receive, store, thaw, and perform other final preparation steps on the cryopreserved product. Feasibility assessment requires consideration of the site's capabilities with regard to specialized personnel, training, equipment, and facilities to execute those tasks.

If cryopreservation is planned as part of the manufacturing process, development teams must consider the effect of cryopreservation on cell number and characteristics and should require reliable methods for cell enumeration and assessment of cell viability and function. During development runs, more assays often are performed than will be required eventually for in-process and final product testing. This is done in order to evaluate the effects of each manipulation of the product. Because some cells may be more susceptible than others to freeze–thaw damage, these studies should include assessment of selective losses of important cell subpopulations within the product.

As described in the *Introduction,* several critical processes influence the outcome of a cryopreservation protocol. Following is a brief discussion of issues unique to cell therapies.

Prefreeze Processing

If cells are harvested with plasma present, samples should be properly processed with an anticoagulant to prevent clotting. In addition, some cells are prone to clumping or aggregation when centrifuged, and some cell products may exhibit excessive damage or loss of one or more populations within the product. Cells harvested from adherent or nonadherent culture may include substantial numbers of dead or fragile cells. Therefore, centrifugation and wash steps should be optimized and specific for the product's cellular contents, suspension volume, suspension medium, and container, along with appropriate evaluation of the cellular product before and after these manipulations. Prefreeze processing should not result in cells that are stressed (e.g., cells that demonstrate elevated early apoptotic markers or temperature-shock responses) before the start of the freezing process, or cell losses will be higher than expected.

Reagents and Containers

Clinical-grade reagents and containers should be used whenever possible (see *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩). It is customary to use sterile, single-use, disposable plastic bags or cryovials that have been qualified for the specific cryopreservation process and subsequent storage conditions. Cryopreservation media for cell therapy products usually consist of isotonic saline-based solutions with one or more CPA, typically the intracellular cryoprotectant DMSO at 5%–10% final concentration with or without an extracellular cryoprotectant such as hydroxyethyl starch. The use of human-derived protein additives such as human serum albumin, serum, or plasma is common, but they may need extensive qualification, so they should be avoided if possible and alternatives should be evaluated. Additives such as animal-derived heparin, citrate-based anticoagulants, and DNase sometimes are used. Many centers formulate their own cryopreservation media, but commercial cryopreservation media, which typically include 5%–10% DMSO and other proprietary components,

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increasingly are used by cell therapy manufacturers to eliminate variability and the need for additional qualification activities associated with local formulation.

Addition of Cryoprotectant Solution and Cooling

Procedures for introduction or removal of a cryopreservation solution should be assessed before freezing to ensure that cell losses resulting from this step are minimized. The cryopreservation medium usually is added to the cell suspension in steps or gradually (e.g., using a syringe pump) to prevent cell losses resulting from osmotic stress. It is common to prechill the cryopreservation medium and keep the cell suspension and the admixture chilled using cold packs, a frozen blanket, or a chilled work surface to prevent heat-related cell damage during addition of DMSO. Once the cryopreservation medium is added, the cell suspension typically is transferred to the precooled chamber of a controlled-rate freezer. During the freeze process, a record of chamber and product temperature over time, or freeze curve, is generated for inclusion in the production record. Product temperatures can be recorded from a probe placed on the product bag's outer surface or from the inside of a comparable product in a dummy bag or vial that undergoes concurrent freezing.

Storage and Transport

Cryopreserved cell therapy products typically are stored and transported at temperatures of –150° or colder. FDA requires screening and testing for evidence of transmissible disease only for allogeneic donors of cell therapy products and not for autologous donations. However, many centers test autologous donors as well and segregate products from autologous donors who are known to have transmissible diseases when their products must be stored. A report of hepatitis B cross-contamination of cellular products within a liquid-nitrogen storage tank led to the currently common practices of storage in the vapor phase of liquid nitrogen and the use of overwrap bags to reinforce product containment. Liquid-nitrogen vapor phase storage may be associated with vertical temperature gradients: products at the top of the storage tank may have a warmer temperature than those stored at the bottom of the tank. Temperature gradients should be monitored, and vertical temperature gradients should be minimized, e.g., by use of metal heat shunts. Overwrap bags may reduce the warming rate of the sample if used during the subsequent thaw process, and their use should be qualified as part of the overall cryopreservation process validation.

Transport of cryopreserved cell therapy products usually is accomplished by using dry shippers containing absorbent material that can be charged with liquid nitrogen to maintain vapor-phase temperatures for up to 2 weeks if properly charged. Data loggers are used to document the temperature history during transport. These shipping containers and procedures must be validated before cell therapy products for clinical use are shipped in the containers.

Warming (Thawing)

Although bedside thawing of cell therapy products before infusion has been a common clinical practice, the use of trained personnel in a controlled laboratory environment is now recognized as the preferred method for thawing because it allows a more standardized process and a higher degree of control when staff must respond to a container failure, which may require product salvage in a more sterile environment. Product thawing typically is done by immersion in 37° water baths and using overwrap bags to minimize product loss and contamination in case of primary container failure. Bags may be gently kneaded during thaw to reduce temperature gradients across the bag and to accelerate thawing. The product is removed from the water bath when some ice is still present in the product but the majority of the product is thawed.

Post-Thaw Processing

DMSO is toxic to cells in liquid suspension. Toxicity may be reduced by diluting or washing the cell suspension before infusion or further manipulation. Because cryopreserved, thawed cells are more sensitive to volumetric expansion when the cells transition from a hypertonic solution to an isotonic solution, the dilution and washing solutions and methods must be carefully designed and validated. Cell washing using either a conventional centrifuge or an automated device can result in additional mechanical stress to the cells, so cell losses must be assessed by an appropriate method before a specific method is implemented in clinical practice.

Quality Control Practices

Quality management of cryopreservation, storage, and thaw of clinical cell therapy products must incorporate quality system elements common to current Good Tissue Practices (cGTP) and current Good Manufacturing Practices (cGMP), including personnel qualification, facility controls, document control, control of equipment and materials, label control, and use of validated SOPs (21 CFR 1271, 210, and 211). Quality control practices specific to clinical cryopreservation typically include assessment and documentation of freeze curves for all products, retention of tubing segments and vials for subsequent testing, and regular monitoring of post-thaw product quality. Practices required by cGTPs and applicable to all human cell- and tissue-based products include measures to ensure accurate and complete labeling and records, to ensure that the correct product goes to the patient, and to allow tracking of the cell product from collection to infusion. The practical implications of these requirements are that labeling and records, including inventory systems, for cryopreserved products must be designed to prevent errors in identification of products. Identity verifications of products moving into and out of cryopreserved storage are performed routinely, e.g., with two people checking the product label against records. If necessary for additional verification of product identity, the contents of a tubing segment attached to the product bag can be thawed and tested before thaw of the entire product. ISBT 128, an internationally recognized system for labeling of blood and cell-therapy products, incorporates use of consistent product nomenclature and barcoding of the product from donor source through administration (see *Appendix*).

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HEMATOPOIETIC STEM CELLS

Studies of the response of hematopoietic stem cells (HSCs) to freezing began in the 1950s, and cryopreserved HSCs have been widely used in clinical practice during the past 30 years. The most common method of cryopreserving HSCs for clinical applications involves the use of 10% DMSO and a controlled-rate freezer set at a cooling rate of 1°/min. Another method, less commonly used, involves the use of passive freezing of the HSC product in a –80° mechanical freezer and the use of 5% DMSO + 6% hydroxyethyl starch solution. Cells commonly are frozen at densities of 30–50 × 10^6 cells/mL. Exceeding cytocrits of 20% (v/v) has been shown to reduce cell recovery. Post-thaw assessment of the sample consists of enumeration of nucleated cells, viable $CD34^+$ cells, and hematopoietic colony-forming units, along with subsequent calculation of recoveries from corresponding prefreeze values.

HSCs cryopreserved for clinical use may be obtained from bone marrow, mobilized peripheral blood, or umbilical cord blood (UCB). Each source has unique requirements for preservation. For example, peripheral blood progenitor cell products contain larger numbers of cells and may be frozen in multiple bags with relatively high cell concentrations. Protocols for UCB preservation may include the use of syringe pumps to introduce cryopreservation solutions while minimizing osmotic stresses. For UCB, specialized solutions often are used after thaw to dilute or remove DMSO while minimizing osmotic stress for the cells.

MESENCHYMAL STEM CELLS

Research on the clinical use of mesenchymal stem cells (MSC) has grown rapidly since the mid-1990s. Reliable, safe, and efficient methods of cryopreservation and storage are critical, especially for allogeneic off-the-shelf MSC products manufactured in multiple product doses for treatment of a large number of patients with a range of clinical indications.

Because MSCs traditionally have been generated in cultures that contain fetal bovine serum (FBS), cryopreservation media for these cells often have incorporated FBS. More recently, alternatives to gamma-irradiated FBS are being explored for the culture expansion before cryopreservation, and cryopreservation has been successful in media that contain 5%–10% DMSO and other components without bovine sources of protein. Although there is no consistent method of post-thaw cell processing, some protocols include dilution or washing of the cells to mitigate the effects of DMSO. Emerging clinical applications for MSCs may require repeat dosing of the cellular product, a practice that requires attention to potential immunogenicity of components of the cryopreservation medium, e.g., FBS or other proteins.

Post-thaw assessment of MSCs typically has involved the use of membrane-integrity dyes such as trypan blue, surface antigen expression, and evidence that the cells are capable of multilineage differentiation. Because the mechanism of action of MSCs may involve the immunomodulatory or trophic properties of the cells, post-thaw assessment also should include relevant cell function.

LYMPHOID CELLS

Lymphocytes are used for a variety of clinical applications including immunotherapy to treat cancer, viral infection, and autoimmune disease. Therapy based on lymphocytes may consist of mixed lymphocyte populations or lymphocyte subpopulations that have been selected or activated ex vivo, e.g., regulatory T cells, natural killer cells, and activated T cells. As with hematopoietic cells, lymphocytes typically are cryopreserved using a 10% DMSO solution and a controlled cooling rate of 1°/min.

Lymphocytes may undergo extensive post-thaw apoptosis, which can influence the clinical efficacy of the cells. Highly purified populations of lymphocytes may exhibit higher levels of post-thaw apoptosis than mixed lymphocyte populations. Strategies such as caspase inhibition and cytokine rescue have been used to diminish post-thaw apoptosis of lymphocytes.

PRESERVATION OF HUMAN PLURIPOTENT STEM CELL LINES

Pluripotent stem cells (PSCs) are cells that appear to have the capacity to (1) undergo self-renewal and replicate indefinitely and (2) generate cells that are representative of the three germ-layer tissues required to create all cells of the human body as demonstrated by the capacity to generate teratomas in immune-deficient mice. The two predominant types of stem cell line used for in vitro laboratory research are human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). hESCs are derived from donated surplus blastocysts by isolation and culture of the blastocyst inner cell mass (the tissue that would have progressed to form the embryo). hiPSCs are created by artificial reprogramming of somatic cells (by the delivery of reprogramming factors using a variety of methods) to yield cells that express the critical properties of PSCs listed above. hiPSCs can be derived from a range of somatic cell types using an increasing range of methods to secure expression of certain reprogramming factors. A range of tissue-derived cultures are known to harbor stem cell populations in vitro (e.g., subcutaneous fat, bone marrow, cord and blood of the fetal umbilicus, primordial germ cells from the fetal neural crest, and neural stem cell spheroid cultures) that have been shown to have a limited capacity for in vitro replication and have not been established as stable diploid PSC lines. Such cultures will not be covered in this chapter, and the following sections will deal specifically with hESC and hiPSC lines.

PSC lines are complex multicomponent cell cultures and may contain a variety of different cell populations with greater or lesser degrees of differentiation or lineage commitment. However, the key property of a stem cell culture, self-renewal, must be sustained. In addition, the cells must retain the capacity to undergo asymmetric division to yield two different daughter cells: one that is a stem cell identical to the parent cell and one with a reduced degree of potency (i.e., ability to generate different cell lineages).

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Development of Current Methodologies for PSC

Currently, individual labs have their preferred methodology, no single approach appears to dominate for routine use, and it seems possible to obtain acceptable levels of post-thaw viability and recovery by either vitrification or controlled slow rate cryopreservation; however, a common method that results in better cell recovery is described here. Briefly, this technique involves placing colonies of hESCs in a vitrification solution composed of 20% DMSO + 20% ethylene glycol (EG) + 0.5 mol/L sucrose after equilibration with lower-concentration DMSO + EG solutions. The colonies are loaded into straws and plunged into liquid nitrogen. Another common method involves placing colonies in a solution consisting of 10% (v/v) DMSO and using either a passive freezing device or a controlled-rate freezer designed to achieve an average cooling rate of approximately 1°/min.

Vitrified IPS or hES samples have special storage and shipping requirements. All cryopreserved or vitrified samples must be stored at temperatures below the glass transition temperature of the sample, and for vitrified samples this is much lower (e.g., −150°) than for traditionally cryopreserved cell samples. In addition, fluctuations in temperatures during storage or shipment can lead to crystallization and therefore degradation of the sample. Because vitrified samples are sensitive to these temperature fluctuations, they should be shipped at liquid nitrogen temperatures and should not be shipped on dry ice.

Challenges for the reliable and reproducible preservation of PSC lines lie not just in the preservation process itself but also in the preparation, cryoprotection, and recovery procedures. Specifically, methods of harvesting and handling before cryopreservation may result in significant cell losses. Processing procedures should be made as reproducible as possible and with the use of an SOP to enhance the reproducibility of preservation outcomes. Furthermore, the effective banking of PSC lines is challenged by the methods by which the cells typically are passaged and harvested for preservation (i.e., individual colonies dissected and transferred as small colony fragments to fresh culture flasks for expansion or to preservation medium for freezing). First, in order to avoid extensive loss of viability in the earlier harvested cells, cultures used to make a bank actually may require preservation in small batches over a working day. Second, preparation of PSC banks comprising smaller pools of cell colony fragments is not only time consuming and laborious, but it also makes it impossible to homogenize the preparation of cells before aliquoting into vials, as would happen with more traditional methods of preserving cell lines. Thus, consistency between vials of PSC lines is compromised. Third, freezing PSCs as colony fragments preserves the gap junctions known to form between cells, potentially leading to intercellular propagation of ice crystals and extensive loss of viability.

To avoid some of these issues, analysts can use enzymatic disaggregation of colonies to simplify and accelerate cell harvesting and to enable cryopreservation of more homogenous, single-cell suspensions before preservation. To be prudent, some laboratories choose to perform enzymatic disaggregation solely before cryopreservation but not for routine culture. Whenever possible, analysts should use non–animal-sourced enzymatic agents for cell dissociation. Another important characteristic of PSC lines is the common but variable incidence of undirected differentiation that occurs within colonies and may vary considerably across the many colonies in a culture. Colonies with a high proportion of differentiated cells should be discarded because the differentiated cells are an undesirable component of a PSC culture and may affect the properties of the undifferentiated cells within the colony.

Points to Consider in the Preservation of PSC Lines

Analysts should consider a number of important factors in the preservation of PSC lines that should be addressed at the levels of preparation, harvesting, banking, and testing of cryopreserved stocks.

EVALUATION AND HARVESTING

Analysts should observe cell cultures on a regular basis. Cultures that exhibit high levels of differentiated cells should be discarded. An individual investigator or banking facility should have a quality control program that evaluates differentiation of cultures on a regular basis and develops threshold levels of acceptable differentiation in a culture. The protocol for harvesting followed by equilibration of the sample with CPAs should be carried out rapidly in order to minimize cell losses because of harvesting.

THE BANKING PROCESS

Conventional cell banking procedures require the development of MCB and WCB. Cells in the MCB should be preserved at an early passage number (P10–P20) in order for experimental and cell line development work to be performed on cells at the lowest possible passage number. The first WCB should be established with the minimum passages to achieve the cell number required. To promote consistency, the WCBs subsequently can be replaced at the same passage level from the MCB as required.

VIABILITY

Determination of post-thaw viability and function for PSCs can be complicated and is often done incorrectly. The most common method of post-thaw assessment is use of a membrane integrity dye. As a measure of viability, the fraction of cells with intact membranes is compared to those without. Another fairly common method is to quantitate PSC colony formations. The total number of colonies seeded into a plate is counted and then is counted again after a certain incubation time. This method also evaluates other functional characteristics of the cells, such as their ability to attach and proliferate. Note that procedures such as harvesting and cryopreservation may induce apoptosis. Staining for early (phosphatidylserine on the cell surface) or later (Annexin VI) apoptotic markers may provide insights into the general health of frozen and thawed cultures.

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HOMOGENEITY

As already described, preservation of colony fragments by vitrification can exacerbate the lack of homogeneity between vials or straws of cells. When larger banks of PSCs are established, analysts should test vials from early, middle, and late positions in the filling sequence of the cell bank for viability, growth rate, and key markers as indicated in *Viability*.

STEMNESS

In order to check that a PSC line has not lost any of its stem cell characteristics during preservation, analysts should check expression of a number of key stem cell-related markers. One extensive study analyzed 59 hESC lines and a panel of 94 genes and resolved five stem cell-related molecules for which mRNA was expressed consistently in hESCs. These genes now are included in commercially available microfluidics gene cards that are specifically designed for investigating stem cell populations. If it is crucial to demonstrate that the PSCs have retained their pluripotency, then a number of characterization tests can be performed, including teratoma formation in immune-deficient mice, formation of trilaminar embryoid bodies, and directed differentiation to demonstrate that the culture can produce representatives of each of the three germ layer tissues that are required to form all of the cells of the human body.

GENETIC STABILITY

In both iPSC and hESC lines, it is not unusual for clones of abnormal karyotype to arise on extended passage and overgrow the culture. Thus analysts should monitor cultures for such abnormal cells. Traditionally, this has been performed by karyotypic studies of metaphase spreads of the cells using Giemsa staining. The occurrence of nondiploid cells, even at very low incidence, can be problematic. Guidance documents such as the 2009 International Stem Cell Banking Initiative are helpful to determine if such cultures should be discarded. However, newer procedures such as array comparative genome hybridization and single-nucleotide polymorphism arrays provide much more detailed analysis of genetic stability and can be used in parallel with Giemsa banding to give greater confidence in genetic stability.

BEST PRACTICES

In addition to following good cell culture practices, analysts should note the availability of a specific guidance that contains principles and best practices in the procurement, banking, testing, and storage of hESCs for research purposes (see the ISCB 2009 reference in the *Appendix*). This guidance is useful for both iPSC and hESC lines.

CELL SUBSTRATES USED IN PRODUCTION AND CHARACTERIZATION OF BIOTECHNOLOGY-DERIVED AND BIOLOGIC THERAPEUTIC PRODUCTS

A wide variety of both recombinant and nonrecombinant cells are cryopreserved and used in the production and characterization of human biologics and biotechnology-derived (B&B) products (more information can be found in ICH Q5D *Derivation and Characterisation of Cell Substrates Used for Biotechnological/Biological Products)*. The major groups include cell lines derived from mammals (including humans), insects (primarily moths), and selected strains of bacteria and yeast. The most common microbial substrates used for production of human biotechnology-derived products are recombinant strains of *Escherichia coli, Pichia pastoris,* or *Saccharomyces* spp. (yeasts). Despite the high degree of diversity among cell types used to manufacture B&B products, there is a surprising degree of uniformity across cryopreservation practices and the same principles apply to cells used for manufacture and those used in tests for adventitious agents and product potency.

Typically, a two- or three-tiered cell banking system is used for maintenance of manufacturing cell lines or microbial strains. For those that use a three-tier system, the first tier bank can be referred to as a research, seed, stock, accession, pre-MCB, or parent cell bank. The source of the pre-MCB can be a research or development laboratory, or cells can be purchased from a commercial repository. It is advisable to characterize the parental cell bank prior to its use in cloning. The second tier (or first tier in a two-bank system) MCB or Master Cell Stock (MCS) is prepared directly from this parent cell bank with minimal cell passages or generations. The MCB or MCS is extensively tested to confirm purity, phenotype, genotype, protein expression, or other important attributes. The WCB or Working Cell Stock (WCS) is derived from vials of the MCB after successive passages in culture. The WCB is the manufacturing cell substrate that is scaled up through repeated subcultures to seed the final production bioreactor, fermenter, or lot of culture vessels (e.g., roller bottles). At each tier in the cell banking system, proper cryopreservation is paramount to success in both product development and manufacturing. In some cases, the end of production cells also may be banked for testing purposes as part of cell bank qualification.

Mammalian and Insect Cell Lines

Mammalian cell lines are the cellular substrates of choice for the production of complex protein molecules. Mammalian cells possess the intrinsic biological machinery required for posttranslational glycosylation of proteins that often is critical for stability and bioactivity in humans. Both diploid and heteroploid cell lines (including hybridomas) are used for production of biotechnology-derived therapeutics. Diploid cell lines are common vaccine substrates [e.g., WI-38 and MRC-5 (human fibroblast cell lines), BHK-21 (baby hamster kidney cell line), and MDCK (Madin-Darby canine kidney)]. Additionally, the African green monkey Vero cell line (a heteroploid cell line) is used for several US-licensed vaccines. Today, commonly used heteroploid cell lines include various recombinant Chinese Hamster Ovary and human embryonic kidney cell lines. Cryopreservation methods are fairly well standardized for these cell lines. However, investigators may find it useful to investigate the toxicity of different cryoprotectants and concentrations when they use a new cell substrate.

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Insect cell lines have proven their capability for production of various recombinant polypeptides. The most common cell lines are derived from the moths *Spodotera frugiperda* and *Trichoplusia ni,* and their established cell lines are called Sf9 and Tn5 (or High Five), respectively. Production of recombinant proteins employs recombinant baculovirus infection for transfer of heterologous genes. Although insect cell lines require different nutritional factors, lower incubation temperatures, and higher osmolarity than their mammalian counterparts, the same essential elements of cryopreservation apply to both groups.

In addition to the guidance given in the *Key Elements of Cryopreservation Practice* section, these additional points should be considered for animal cell line substrates:

PREFREEZE PROCESSING

Analysts should ensure that cells are not contaminated and are still viable. For diploid cells, analysts should grow to a passage level to maintain diploidy and below the intended level for use. Analysts pool cell cultures and perform a cell count to determine the number of viable cells available for banking. Harvested cells are centrifuged at a relatively low speed for a short duration, e.g., 100–200 × *g* for 5–10 min, preferably using a refrigerated centrifuge.

CPAS AND CRYOCONTAINERS

The membrane-permeable CPA of choice is 5%–10% (v/v) DMSO diluted in fresh growth medium. For certain sensitive cell lines, cell culture-conditioned medium can be added to supplement the cryopreservation medium. DMSO must be sterile and tissue-culture grade (>99% purity). The most appropriate cryocontainer is a presterilized polypropylene screw-cap vial designed for cryogenic storage in vapor phase liquid nitrogen.

INTRODUCTION OF CRYOPRESERVATION MEDIUM

Immediately following centrifugation of cells, growth medium is removed from cell pellets, and cells are gently resuspended by slow addition of cryopreservation medium that is often precooled for many cell types. The cell suspension is immediately diluted with an appropriate volume of cryopreservation medium based on viable cell count and targeted cell density (typically, cell banks are produced at a viable cell density of approximately 1×10^7 cells/mL). As mentioned in the previous section, cryopreservation medium is highly hypertonic, and exposure time should be limited. The final cell suspension is transferred to a vessel in which the cells can be gently mixed during vial filling to facilitate uniformity of the cell bank.

COOLING AND CONTROLLED FREEZING AND STORAGE

Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine. In either case, vials are usually refrigerated as the filling progresses in order to minimize potential toxic effects of DMSO at higher temperatures. Time limits should be established for the entire filling process and recorded in the batch record. Immediately after the vials are filled, analysts should transfer them into a controlled-rate freezer or, alternatively, into an ultracold static freezer (e.g., –80°) using an insulated container designed for controlled cooling. Any controlled freezing system should be properly qualified to ensure expected cooling rates are delivered to all vials within the load. When using a controlled-rate freezer, analysts determine optimal cooling programs empirically, but a rate in the range of 1°–5°/min after transition through heat of fusion should be acceptable for most cell lines (colder than about –40°, the rate can be increased, e.g., 10°/min). After vials have been frozen to approximately –80° or below in the freezing system, they are transferred as quickly as possible to liquid-nitrogen vapor phase storage units.

WARMING AND VIAL THAWING

In general, cells frozen at a slow cooling rate should be thawed as quickly as possible to maximize cell viability. In order to ensure uniformity of temperature, vials should be transferred directly from liquid-nitrogen vapor phase storage into a portable vapor phase (dry) shipper for transport to the laboratory. If a liquid-nitrogen Dewar is not available, then vials can sometimes be packed in dry ice, but this process should be demonstrated as suitable because it can result in detrimental pH changes. After transfer to the laboratory, vials are placed directly into a warm water bath (e.g., 37°, making sure caps are not immersed), a bead bath, or a thermoblock. Note that insect cell lines should be thawed at 27°–30°. Vials should be agitated to facilitate uniform thawing of cells. Immediately after thawing, vials should be transferred into the biological safety cabinet and sanitized before opening. Typically, growth medium is slowly added to thawed cells while agitating to dilute DMSO and to slowly reduce the osmolarity of the post-thaw milieu back to a physiological level.

POST-THAW PROCESSING

Different methods can accomplish this step, but analysts should achieve sufficient dilution of the DMSO and return the cells to their normal isotonic growth environment. Manipulation of cells immediately post-thaw should be minimized because of the stresses induced by the freeze–thaw process. For example, pipetting and centrifugation should be minimized.

Microbial Strains: *E. Coli,* Yeast, and Bacterial Vaccine Strains

Recombinant *E. coli* strains have a proven track record for production of a number of commercially viable biotechnology-derived products, including recombinant insulin analogs, human growth hormone, and parathyroid hormone. Compared to their mammalian and insect counterparts, recombinant *E. coli* strains are relatively simple to grow and scale up to large volumes, e.g., 40,000 L. However, bacteria lack the sophisticated cellular machinery for building more complex protein molecules that require post-translational modifications such as glycosylation. Yeasts are unicellular, eukaryotic cells that can be

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manipulated genetically to produce a wide range of recombinant proteins and peptides with limited complexity. Despite the evolutionary distances between the mammalian and microbial cell substrates, the same set of essential cryopreservation elements described above apply, with the following unique points:

PREFREEZE PROCESSING

Cultures should be propagated in shake flasks to late logarithmic or early stationary phase using strain-specific growth conditions.

CPAS AND CRYOCONTAINERS

The cell wall or cell membrane-permeable CPA of choice is glycerol (although DMSO is also sometimes used) at concentrations typically ranging from 5%–10% (v/v). Synthetic glycerol should be used for registration of commercial products along with other raw materials that are free of animal components.

INTRODUCTION OF CRYOPRESERVATION MEDIUM

Because microbial cells possess cell walls for protection and support of cytoplasmic contents, physical manipulation and osmotic shifts do not have the same negative impact seen in animal cell lines. Immediately after centrifugation, cell pellets are vigorously resuspended in the cryopreservation medium (to a dilution based on requirements of the cell bank for number of vials and viable colony-forming units/mL). This dilution step can be based on active measurement of culture optical density or other cell enumeration assays. The final cell suspension is transferred to a vessel in which the cells can be mixed during filling of final containers to facilitate uniformity of the cell bank. Because of potential toxicity, analysts should limit the time of exposure to glycerol, despite the relative robustness of microbial cells. Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine.

COOLING AND CONTROLLED FREEZING AND STORAGE

Again, because of their inherent robustness, microbial cells do not require as strict control of cooling rate as do animal cell lines. Consequently, the choice of freezing system or method has a smaller effect on the viability of the cell bank. Filled cryocontainers simply can be transferred into an approximately –80° freezer overnight followed by transfer to vapor phase liquid nitrogen. Alternatively, microbial cell banks may not use liquid nitrogen storage but can be stored at approximately –80°. However, if liquid-nitrogen storage is available, it is preferred for long-term storage (e.g., years). If a controlled-rate freezer is employed, then it should be fully qualified to deliver a uniform cooling rate that is within an expected range, e.g., 1°–5°/min.

WARMING AND CONTAINER THAWING

Controlling the rates of cooling and warming is less critical for microbial cells, but the cells still should be thawed as quickly as possible using a warm water bath (e.g., 30°–35°). Temperatures can be adjusted to the incubation temperature of the strain.

POST-THAW PROCESSING

Growth medium is added to dilute the thawed cells to a desired level of colony-forming units/mL and to induce removal of intracellular glycerol.

All cells are not created equal and are divergent by nature. A broad span of evolutionary time separates the various cell substrates used for manufacture of human B&B products. Fortunately for the practitioner of industrial cell culture, cryopreservation strategies converge to a set of shared principles and methods that translate across evolutionary paths.

APPENDIX

Useful Guidances

- FDA. Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. 2010. http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf. Accessed 24 September 2012.
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Change to read:

⟨1046⟩ ▲CELL-BASED ADVANCED THERAPIES▲ (USP 1-May-2020) AND TISSUE-BASED PRODUCTS

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INTRODUCTION

This general chapter provides a comprehensive overview of considerations for the development of ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue-based products. A collection of terms commonly used in this field is provided in *Glossary*. ▲Cell-based advanced therapies▲ (USP 1-May-2020) and tissue-based therapies are medical products that contain human or animal cells that will be administered to humans to repair, replace, regenerate, or augment a recipient's cells, tissues, or organs that are diseased, dysfunctional, or injured. The source cells or tissues can be harvested for use without manipulation or may be propagated, expanded, pharmacologically treated, or otherwise altered in biological characteristics ex vivo before administration. The diversity of clinical indications and types of ▲cell-based advanced therapies▲ (USP 1-May-2020) are shown in *Table 1*. ▲Examples of tissue-based products are included in *Table 2*.▲ (USP 1-May-2020)

Table 1. Examples of Cell-Based ▲Advanced Therapies

Disease/Condition	Example▲ (USP 1-May-2020)
Hematopoietic stem cell transplantation following ablative therapy	Hematopoietic stem and progenitor cells that have been harvested, propagated, selected, and/or treated for removal of contaminating cells by means of devices and/or reagents
Cancer	T cells, NK cells, dendritic cells, or macrophages ▲targeted▲ (USP 1-May-2020) to cancer-specific peptides to elicit an anticancer response; autologous or allogeneic cancer cells, genetically or biochemically modified and irradiated to elicit an anticancer response
Diabetes	Encapsulated β-islet cells
Myocardial infarction	Autologous or allogeneic stem/progenitor cells; skeletal myocytes; cardiac-derived stem cells
Graft-versus-host disease	Allogeneic mesenchymal stem cells
Wound healing	Autologous keratinocytes or allogeneic dermal fibroblasts on a biocompatible scaffold
Focal defects in knee cartilage	Autologous or allogeneic chondrocytes with or without a biocompatible scaffold
Bone repair	Mesenchymal stem cells in a biocompatible scaffold
Neurodegenerative diseases	Neuronal progenitor cells derived from embryonic, fetal, or adult source tissues; cells genetically modified to secrete neurotrophic factors, with or without encapsulation
Infectious disease	Activated T cells
Autoimmune disease	Regulatory T cells (T_{reg})
Spinal cord injury	Neuronal progenitor cells
Organ repair or regeneration	Autologous or allogeneic cells on biocompatible biomaterials (gels) or 3-dimensional scaffold structure

Cell therapy products can be modified by treatment with integrating or non-integrating genetic materials (DNA, RNA, siRNA, etc.) so that the pattern of gene expression is changed. Typically, cells are taken from the patient or a healthy donor, and are modified outside of the body before they are given to the patient. Regulatory bodies consider the ex vivo gene-modified cellular product to be a gene therapy product. A great deal of information in this chapter is relevant to processing, characterization, manufacturing, and administration of genetically modified cells. However detailed information about the use of various gene transfer systems, patient monitoring considerations, genetic analysis, and other issues pertinent to gene therapy products are addressed in *Gene Therapy Products* ⟨1047⟩.

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▲Table 2. Examples of Tissue-Based Products

Uses	Example Products
Musculoskeletal	Decellularized, freeze-dried demineralized human bone (cortical or cancellous)
	Decellularized, freeze-dried human bone (cortical or cancellous)
	Decellularized human dermis
	Decellularized bovine dermis
	Decellularized porcine dermis
	Decellularized porcine subintestinal submucosa (SIS)
	Human cancellous bone with adipose-derived stem cells
	Human demineralized cortical bone and cancellous bone with bone marrow-derived stem cells
Neurological	Decellularized human nerve tissue
Ophthalmology	Human amniotic tissue
	Human placental tissue
Wound care	Decellularized human dermis
	Decellularized porcine SIS
	Human amniotic tissue
	Human placental tissue

For tissue-based grafts that contain non-autologous cells, the cells are derived from the same donor; they may be harvested with the tissue, or harvested from a different organ (e.g., bone marrow aspirate, adipose) and recombined with the tissue (e.g., bone).▲ (USP 1-May-2020)

This chapter describes issues related to the manufacturing, sourcing of components, and characterization of ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based products to ensure their safety and efficacy. A list of relevant regulatory and guidance documents is presented in the *Appendix*. Manufacturers of ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based products should consider and apply the controls and procedures outlined in this chapter to ensure the products' safe use in humans. New methodologies are continually being developed and validated and will be included in the United States Pharmacopeia (*USP*) as they become available. *USP* monographs for specific tissue and tissue-based products outline test specifications that should be met throughout a product's time in the market place. The term "cellular ▲therapy▲ (USP 1-May-2020) product" refers to living human or animal cells or tissues that ▲are subject to approval▲ (USP 1-May-2020) by the US FDA. ▲This generally means clinical trials are required to demonstrate safety and efficacy prior to commercialization under a biologics license application (BLA), or for a medical device, premarket approval (PMA). Additionally, most cellular therapy products must comply with both good tissue practices (GTPs) and good manufacturing practices (GMPs). A subset of cell-based products, known as combination products, or tissue engineering products, refer to cells combined with a medical device, such as a natural or synthetic scaffold. These products must also meet similar standards for establishing safety and efficacy as well as comply with GTPs and GMPs, or medical device quality system regulations (QSR). Most cell-based advanced therapies and tissue engineering products are likely to fall within the legislation that defines regenerative medicine advanced therapies (RMAT). The majority of commercially available tissue-based products are human derived. Human tissue-based materials are often referred to as HCT/Ps, or human cells, tissues, cellular and tissue-based products. HCT/Ps that meet criteria identified in 21 Code of Federal Regulations (CFR) 1271.3(d)(1) and Section 361 of the Public Health Service (PHS) Act do not require premarket approval by FDA (i.e., BLA or PMA) and only need to comply with GTP regulations. These "361 products" meet criteria for "minimal manipulation", and are utilized in applications consistent with "homologous use". Human tissue products not meeting these criteria may require premarket clearance [510(k)] or PMA or BLA. While less common, some tissue-based materials are animal derived and generally require premarket clearance [510(k)].▲ (USP 1-May-2020)

Considerations for Incorporating Quality System Concepts Early in ▲Cell-Based Advanced Therapies▲ (USP 1-May-2020) and Tissue-Based Product Development

Current and future regulatory requirements will continue to challenge developers of ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue-based products to incorporate robust quality attributes early in the design phase to ensure a focus on patient safety by means of a high degree of process understanding. Modern quality systems that harmonize current Good Manufacturing Practices (cGMPs) with other non-US pharmaceutical regulatory systems [such as the International Council for Harmonisation (ICH) and the ISO] and the FDA medical device quality system are being recognized ▲for ensuring quality.▲ (USP 1-May-2020) These new standards include product development concepts such as Quality by Design (QbD) and Process Analytical Technology (PAT). Moreover, these quality systems integrate approaches to continual improvement and risk management that promote adoption of the latest scientific advances and innovative manufacturing technologies.

Employing the principles of Quality Risk Management (QRM) early in product development may identify areas of risk that can be mitigated before they are incorporated into the manufacturing process and affect the safety and efficacy of the product.

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Developers of ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue-based products should employ risk management and assessment techniques as a key component of their quality systems. "Risk management" is defined as a systematic process for the identification, assessment, and control of risks to the quality of the ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based product across the product lifecycle. Using QRM techniques can help achieve safe and efficacious products by assessing patient risks, determining design space boundaries, or ranking quality attributes. QRM can also establish and maintain a state of control by using risk management to drive process control. Finally, QRM can be used to facilitate continual improvement by prioritizing opportunities for improvement. The level of effort, formality, and documentation of the risk management process should be commensurate with the level of risk, should be based on scientific ▲and medical▲ (USP 1-May-2020) knowledge, and ultimately should be linked to patient protection.

The elements of risk management have become an accepted paradigm; ▲these are described▲ (USP 1-May-2020) in FDA and international regulatory guidance documents, especially ICH Q9. A number of tools have been developed to facilitate this assessment. These tools provide a quantifiable means of prioritizing risk so that higher-risk elements of a process can be identified and corrected.

Depending on the objective of the risk management program, risk analysis can be more or less formalized. ▲▲ (USP 1-May-2020) A more formalized risk assessment system is necessary for process or product development. This is especially important when limited resources must be prioritized. Formalized systems are predicated on well-established tools that can quantify risk in every phase or step of manufacturing. These systems can also be used in evaluating raw material choices, validation prioritization, facility alterations, equipment changes, and utility deliberations.

Formal risk analysis tools include process mapping, preliminary hazard analysis, Hazard Analysis of Critical Control Points (HACCP), Hazard Operability Analysis (HAZOP), Fault Tree Analysis (FTA), Failure Mode Effects Analysis (FMEA), and Failure Mode Effects and Criticality Analysis (FMECA).

For ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue-based products, FMEA has been commonly used to identify, quantify, and prioritize risk. FMEA can assign a numerical rating in one of three categories:

- "Severity", which is the consequence of a failure;
- "Occurrence", which is the likelihood of the failure happening based on past experience or nonconformance; and
- "Detection", based on the ability to detect the failure.

Each category is assigned a numerical rating (typically 1–5 or 1–10) consistent with the severity of the excursion from the operating parameter range, the probability of an excursion, and the likelihood of detecting an excursion before it has an effect on the product. Lower numbers refer to an unlikely probability of detection whereas higher numbers refer to the likelihood of a failure or hazardous effect. The product of the severity, occurrence, and detection values is a Risk Priority Number (RPN). In the risk-evaluation process RPNs are prioritized, and the most immediate remediation can be directed to areas of highest risk.

Change to read:

COMPONENTS USED IN CELL▲-BASED ADVANCED THERAPIES▲ (USP 1-MAY-2020) AND TISSUE-BASED PRODUCT MANUFACTURING

Introduction

Manufacturers of ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based products must ensure that all components used in manufacturing are appropriately qualified. Examples of components used in the production of ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based therapies include the source cells and tissues; natural or synthetic biomaterials; ancillary materials required during manufacturing but not intended to be present in the final therapeutic product; and excipients used in the formulation of ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based therapies. ▲For more information on ancillary materials, see *Ancillary Materials For Cell, Gene, And Tissue-Engineered Products* ⟨1043⟩.▲ (USP 1-May-2020)

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific component to ensure quality. The diversity of ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue-therapy products and the materials used to produce them makes it difficult to recommend specific tests or protocols for a qualification program. Therefore, rational and scientifically sound programs must be developed for each component.

Material qualification activities will change as products move from clinical trials to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety concerns should be the primary focus of a material qualification plan. In the later stages, material qualification activities should be completely developed and should comply with cGMP.

Qualification of Source Cells and Tissues

Various human- and animal-derived cells and tissues serve as source material for cell and tissue-based products. Three sources of donor cells for cell-therapy products include:

1. The patient's own cells (autologous cell products)
2. Cells from another human being (allogeneic cell products)
3. Cells derived from animals (xenogeneic cell products)

The source of cells used for a particular cell or tissue-based therapy largely depends on compatibility, purity, and availability. Use of autologous cells has the advantage of minimal concerns regarding immune rejection. However, an autologous source is not always available and appropriate if the cell type is dysfunctional, malignant, not readily obtainable, or contaminated.

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The alternative is a compatible allogeneic cell source that may be more readily available. Of primary concern with the use of allogeneic cell sources is immune incompatibility, which could lead to rejection of the administered cell- or tissue-based therapy. In immunocompromised recipients, the donor cells may react to the patient's cells, leading to graft-versus-host disease, which can be life threatening. Despite the potential complications of using allogeneic donor cells or tissues, in the absence of other alternatives the risk-to-benefit ratio ▲may be acceptable.▲ (USP 1-May-2020) A number of approaches successfully circumvent immune barriers for the use of allogeneic sources. Immunosuppressive drugs developed for solid organ transplantation and advances in inducing immune tolerance are increasingly applied to cell transplantation. Certain allogeneic cells elicit minimal immune reactions, even in human leukocyte antigen (HLA)-mismatched recipients. Examples include mesenchymal stem cells, certain dermal and epidermal cells, and fibroblasts. ▲Cells may be obtained from living or deceased donors.▲ (USP 1-May-2020)

Despite advances in the derivation of new types of therapeutic cells, particularly stem cells (adult, fetal, embryonic and induced pluripotent cells), the ability to generate certain types of cells or tissues remains elusive. As a result, ongoing efforts use xenogeneic cells and tissues to treat a number of human diseases or conditions. Use of xenogeneic cells ▲and tissues▲ (USP 1-May-2020) must address concerns about both immune rejection and transmission of animal viruses to humans (see *Animal Sources of Cells and Tissues* below). ▲Both human- and animal-sourced cells and tissues can pose a risk for transmission of adventitious agents.▲ (USP 1-May-2020)

Some general principles in the sourcing of tissues include: 1) systems must allow the material to be traced back to the donor, while adhering to privacy legislation; 2) steps must be taken to prevent the transmission of infectious diseases from the donor to the recipient; and 3) ▲while terminal sterilization is employed for some decellularized tissue-based materials,▲ (USP 1-May-2020) aseptic procurement and processing must ensure the safety of the final product because terminal sterilization of products containing living cells and tissues is not possible. The FDA has promulgated a specific set of regulations, referred to as GTPs, that specifically address the need to procure and process tissues in a manner that avoids transmission of a communicable disease. GTPs and/or GMPs must be followed for ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based therapy products, depending on cell source.▲ ▲ (USP 1-May-2020)

DONOR ELIGIBILITY

FDA has enacted a comprehensive set of regulations governing human tissues and human cells that are intended for implantation, transplantation, infusion, or transfer into a human recipient. These materials are referred to as HCT/Ps. Paramount for procurement of HCT/Ps for medical use is adherence to donor eligibility requirements. These dictate that a donor's relevant medical records must be reviewed to evaluate risk factors and clinical evidence of communicable disease agents. This includes obtaining a health history and ▲could include▲ (USP 1-May-2020) performing a physical ▲assessment▲ (USP 1-May-2020) on a donor to screen for communicable diseases. In addition, donors must also undergo appropriate laboratory testing using FDA-cleared or -approved test kits for specific relevant communicable disease agents and diseases (RCDADs). Required disease testing will expand as new RCDADs are identified and FDA-cleared or -approved ▲tests or▲ (USP 1-May-2020) test kits become available. ▲▲ (USP 1-May-2020) Sources for information about communicable disease testing are FDA's *Guidance on Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, ▲American Association of Tissue Banking (AATB) Standards, and subsequent recommendations for emerging RCDADs such as West Nile Virus and Zika Virus are available from the FDA website (www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/default.htm).▲ (USP 1-May-2020)

HUMAN CELLS, TISSUES, OR ▲CELL-BASED ADVANCED THERAPIES▲ (USP 1-May-2020) OR TISSUE-BASED PRODUCTS

HCT/Ps may be sourced from normal healthy donors, cadaveric donors, or patients with diseases such as cancer. The suitability of tissue sourced from patients with cancer and other diseases should be assessed before collection to ensure adequate safety and function of the final cell therapy product. Additionally, the regulations in 45 CFR Part 46 apply to all federally supported human subject research. These regulations require that an Institutional Review Board review and approve the use of any tissue taken from a human donor. The regulations also include special considerations for research on prisoners, children, pregnant women, or gestational tissue. In all cases appropriate written consent must be obtained from the donor or the donor's next of kin describing the tissue that is being procured and its intended use.

The risk of disease transmission to the manufacturing operator should be minimized by appropriate training for handling potentially infectious materials and by the use of protective equipment and clothing. Tissues should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery.

Hematopoietic progenitor cells (HPCs) are one of the most extensively used cell sources for human transplantation. These cells can be collected from the bone marrow, peripheral blood, or umbilical cord blood. The source of cells depends on the patient, the disease, and the clinical protocol. Regardless of the cell source, methods for processing the cells are similar. HPCs can be sourced from healthy donors or patients with hematological disorders. In addition to FDA's HCT/Ps regulations, applicable guidelines and standards for the collection and processing of these materials have been published by the American Association of Blood Banks (AABB), the Foundation for the Accreditation of Hematopoietic Cell Therapy, and the National Marrow Donor Program (NMDP).

For cell or tissue sources obtained from surgical specimens or cadaveric donors, standard hospital operating room practices are applicable. The air quality in a typical limited-access operating room is adequate for such procedures. Procurement personnel must be appropriately trained in all aspects of tissue recovery, such as surgical scrubbing, gowning, operating room behavior, anatomy, surgical site preparation, and aseptic technique. Special care is required when tissue or organ procurement requires extensive manipulation of the bowel, which may result in the inadvertent puncture of the bowel. ▲Consideration should be given to sampling incoming tissues for microbial bioburden. Though it is recognized that results of such bioburden testing would likely not be available until after the tissue has been processed, in the event of a subsequently observed microbial

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contamination during downstream processing or positive sterility result, the bioburden results from the incoming tissue could aid in the investigation of the source of the contamination.▲ (USP 1-May-2020)

ANIMAL SOURCES OF CELLS AND TISSUES

Ideally, cellular therapy products would consist of human cells manufactured with minimal exposure to animal-based materials. However, at present important unmet medical needs may potentially be addressed by cellular therapy products from animal cells or tissues. One example is pancreatic islets intended to treat diabetes. ▲Currently▲ (USP 1-May-2020) human sources of pancreatic islets are available only from pancreas donated at the time of death. The quality of donor organ islets is variable, and the available supply is inadequate to meet potential demand. One approach is procurement of pancreatic islets from appropriately qualified animal sources for subsequent use in humans (xenotransplantation).

Developers who intend to use ▲viable▲ (USP 1-May-2020) animal cells or tissues in a ▲cell-based advanced therapy or tissue-based▲ (USP 1-May-2020) product must adequately address public health concerns and must develop approaches to mitigate the potential risk of introduction and propagation of zoonotic infectious agents into the general human population. The *PHS Guideline on Infectious Disease Issues in Xenotransplantation* (January 2001) describes potential risks. The FDA guidance *Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans* (April ▲2016▲ (USP 1-May-2020)) reflects updated approaches and expectations to minimize risks of xenogeneic cellular products ▲(https://www.fda.gov/regulatory-information/search-fda-guidance-documents/source-animal-product-preclinical-and-clinical-issues-concerning-use-xenotransplantation-products). In most cases, decellularized (i.e., nonviable) xenograft tissue-based products are not subject to the PHS guideline and FDA guidance mentioned above and are generally regulated in the US as medical devices and must follow the applicable regulatory pathway [e.g., 510(k)] and all related requirements.▲ (USP 1-May-2020)

The use of ▲viable▲ (USP 1-May-2020) animal tissue in the manufacture of ▲cell-based advanced therapies▲ (USP 1-May-2020) requires that the tissue be sourced in a controlled and documented manner from designated pathogen-free animals bred and raised in captivity in countries or geographic regions that have appropriate disease prevention and control systems. In addition, the care and use of animals should be approved by a certified institutional animal care and use committee. Donor animals must have documented lineage, be obtained from closed herds or colonies, and be under health maintenance and monitoring programs. The animal housing facility should be USDA certified (large vertebrate animals) or Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) certified (small vertebrate animals) and should meet the recommendations stated in the ▲current version of the▲ (USP 1-May-2020)*Guide for the Care and Use of Laboratory Animals* (National Research Council▲▲ (USP 1-May-2020)), which can be obtained from AAALAC (www.aaalac.org). Such facilities should be staffed with veterinarians and other trained personnel who ensure animal health and disease prevention. The facility's procedures should be documented, and records should be kept. Health maintenance and monitoring programs should be based on standard veterinary care for the species, including physical examinations, monitoring, laboratory diagnostic tests, and vaccinations. A stepwise "batch" or "all-in–all-out" method of source animal movement through the facility can minimize the potential for transmission of infectious agents.

Feed components should be documented and should exclude recycled or rendered materials in order to reduce the risk of prion-associated diseases.

To provide a high degree of assurance of product safety, animal donors and tissues should be screened at several stages throughout the process to rule out the presence of microbial agents. These control tests should utilize assays that are sufficiently sensitive and specific to detect bacteria, mycoplasma, fungi, or viruses of interest. Donor animals should be screened for relevant diseases before tissue procurement. Post-tissue-retrieval necropsies, sentinel animal programs, and archival storage of donor organs, tissues, blood, and other specimens also ensure the safety of animal tissue for use in cellular therapeutic applications.

In general, similar aseptic procurement issues apply to animal and human tissues. The tissue should be obtained under environmental conditions and controls that provide a high degree of assurance of aseptic recovery. Specifically designed procurement facilities, usually closely associated with the animal holding facility, should be employed. Recommended design features and attributes of the animal tissue procurement facility should include the following: 1) staging of events such as shaving, sedation, and operating room preparation in separate rooms with appropriate environmental controls; 2) high-efficiency particulate air (HEPA) filtration; 3) adjacent but separate facilities for further tissue processing; and 4) dedicated areas for carcass removal. Issues relating to personnel training, bowel manipulation and puncture, and disinfection apply to the surgical procurement of both human and animal tissues (see *Human Cells, Tissues, or* ▲*Cell-Based Advanced Therapies*▲ (USP 1-May-2020) *or Tissue-Based Products* above). When researchers establish animal cell lines for use ▲in production,▲ (USP 1-May-2020) cell banks should be created, tested, and characterized as described in the next section.

CELL BANK SYSTEM

A cell bank is a collection of cells obtained from pooled cells or derived from a single cell clone or donor tissue that is stored in bags or vials under defined conditions that maintain genotypic and phenotypic stability. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although alternative approaches are possible. The MCB is produced in accordance with cGMP and preferably is obtained from a qualified source ▲▲ (USP 1-May-2020) with known and documented history. Human cells and tissues should be obtained by means of a licensed tissue acquisition vendor with a donor qualification program in accordance to 21 CFR 1271. The WCB is produced or derived by expanding one or more vials of the MCB. The ▲WCB or MCB▲ (USP 1-May-2020) becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to production batch consistency because the starting cell material is always the same. However, it may not be possible or feasible to create a cell bank, so appropriately tested and qualified primary cells may be used in lieu of creation of cell banks. The MCB and WCB should ▲at a minimum be▲ (USP 1-May-2020) tested for identity, sterility, purity, viability, and the presence of viruses and mycoplasma.

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CELL BANK QUALIFICATION

Cell bank safety testing and characterization are important steps toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. ICH Q5A, *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*, gives specific recommendations for testing cell banks for viral agents. While this guideline is not specifically intended to cover cell- or tissue-based products, the same tests are generally applicable. Additional virus testing may be needed depending on the prevalence of viral diseases endemic in the donor population. Testing to qualify the MCB is performed once and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB should be prospectively established. It is important to document the MCB history, the methods and reagents used to produce the bank, and the storage conditions. All the ancillary materials required for production of the banks, such as media, sera, cytokines, growth factors, and enzymes, should also be qualified, documented, and appropriately tested.

SAFETY TESTING OF MCB AND WCB

Master cell bank: Safety testing to qualify the MCB includes testing to demonstrate freedom from adventitious agents and endogenous viruses. The testing for adventitious agents should include tests for bacteria, fungi, mycoplasma, and viruses. Freedom from adventitious viruses should be demonstrated using both in vitro and▲/or▲ (USP 1-May-2020) in vivo test systems and appropriate species-specific tests,▲demonstrated using both in vitro and/or in vivo test systems, such as appropriate species-specific tests, massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR), hybridization to oligonucleotide arrays, or mass spectrometry, as allowed by regulatory authorities.▲ (USP 1-May-2020)
Working cell bank: Safety testing of the WCB is less extensive and generally focuses on the potential for introduction of adventitious viruses or activation of latent virus during the additional culture required to create the WCB. End-of-production (EOP) safety testing should also be performed to ensure that the cells can be expanded a known maximum number of generations while still producing an acceptable product. For information about which types of adventitious virus testing should be performed on the MCB, WCB, and EOP cells, consult *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

CHARACTERIZATION OF MCB AND WCB

Characterization of the MCB and WCB includes identity testing to establish species origin, e.g., isoenzyme analyses to confirm the human origin of the cells. However, cell bank characterization should encompass additional assessments such as the following:

- Growth kinetics and population doubling time
- Morphological assessment
- Percent confluence at passage
- Cell counts
- Viability (pre- and postcryopreservation)
- Phenotypic expression of desired and undesired cell types (pre- and postcryopreservation)
- Monitoring of unique biochemical markers (pre- and postcryopreservation)
- Assessments of functional activity (pre- and postcryopreservation)
- Gene and protein expression analysis (pre- and postcryopreservation)
- Expression of immune histocompatibility antigens (HLA/M HC)
- Molecular fingerprinting
- Chromosomal stability

Biocompatible Scaffold Materials

▲As previously described,▲ (USP 1-May-2020) most natural or synthetic scaffold materials are regulated as medical devices, although scaffolds derived from human tissues ▲that meet minimal manipulation and homologous use criteria (e.g., demineralized cortical bone particles, or cancellous bone matrix granules) are regulated as "361 tissue" HCT/Ps.▲ (USP 1-May-2020) When ▲suitable,▲ (USP 1-May-2020) use scaffolds that have previously been approved for other clinical uses because these materials should have already undergone extensive safety and quality testing. For applications in cell or tissue-based products, the scaffold material should allow cells to attach, proliferate, and migrate, and high porosity is often desired to facilitate cell seeding within the material. The scaffold must provide adequate diffusion of nutrients for cell health and release of cell-excreted products. ▲Depending on the intended use, the material should have adequate mechanical strength and be▲ (USP 1-May-2020) amenable to manipulation, chemical modification, and manufacture. The scaffold material should be biocompatible, relatively inert, and immunologically benign.

Scaffolds can generally be classified as hard or soft. Hard scaffolds are used in applications where a specific shape is required, such as forming a blood vessel or a bladder. Soft scaffolds are used in applications where the product needs to conform flexibly to an existing shape in the body.

Scaffold materials can be synthetic or natural polymers, biodegradable or permanent. Biodegradation allows the scaffold to be resorbed or removed from the body without manipulation ▲and for the defect to be filled with viable tissue.▲ (USP 1-May-2020) The scaffold degradation rate must coincide with the rate of formation or regeneration of the tissue. The natural scaffold structure must replace the degrading scaffold in such a way that it maintains the structural integrity of the tissue or organ being

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regenerated. For example, a newly formed blood vessel must withstand both the internal blood pressure as well as external mechanical forces.

The most commonly used synthetic biodegradable polymer is polyglycolic acid (PGA). Polylactic acid (PLA) is also widely used, sometimes in combination with PGA. These polymers degrade within the body, are readily removed before degradation, and have a long history of use in suture materials. ▲Polycaprolacton (PCL),▲ (USP 1-May-2020) which exhibits a slower rate of degradation than PLA or PGA, is used in applications that require a long presence in the body.

Extracellular matrix (ECM) and its derivatives are natural materials used for scaffolds in the manufacture of cell–biomaterial combination products. ▲Example sources of ECM include dermis, subintestinal submucosa (SIS), pericardium, and other predominantly collagen containing organs.▲ (USP 1-May-2020) Proteins such as collagen or fibrin and polysaccharides such as chitosan or glycosaminoglycans (GAGs) have also been used in growing cells to make combination products. Collagen is by far the most popular substrate for cells and has been molded into scaffolds for a variety of products, mainly in tissue-engineered skin applications. Cross-linking agents such as glutaraldehyde and water-soluble carbodiimides have been used to enhance the strength of natural scaffolds. Depending on the source of the material, ▲and the extent of decellularization,▲ (USP 1-May-2020) natural scaffolds can be immunogenic.

▲▲ (USP 1-May-2020)

The safety and biocompatibility of the scaffold and product-contact materials must be established. A full battery of tests ▲should be performed as▲ (USP 1-May-2020) recommended by *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, ▲ISO 10993-1, or FDA Guidance: Use of International Standard ISO 10993-1.▲ (USP 1-May-2020) Process residuals and degradation products from the preparation of the scaffold should be quantified and limits should be established. The stability and storage conditions of scaffold materials should be established.

Qualification of Ancillary Materials

Ancillary products include a wide variety of raw materials and components used in manufacturing, ▲but are not intended to be present in the final therapeutic product.▲ (USP 1-May-2020) They ▲▲ (USP 1-May-2020) include ▲▲ (USP 1-May-2020) substances such as culture media, buffers, growth factors, cytokines, cultivation and processing components, ▲monoclonal antibodies, and components of cell-separation systems.

Residual ancillary materials may be antigenic, so their removal from the final product should be assessed, and appropriate limits should be established when necessary.

Ancillary material quality can profoundly affect the safety, potency, and purity of the cellular or tissue-based product. Ideally, each ancillary material should be produced under conditions that are in compliance with cGMP. However, some complex or unique substances may not be available from cGMP–compliant suppliers. In these situations, the cellular or tissue-therapy product manufacturer should develop a scientifically sound strategy for qualifying the ancillary material. Such a qualification program should address each of the following areas: 1) identification and selection, 2) suitability for use in manufacturing, 3) characterization and acceptance criteria, 4) vendor qualification, and 5) quality assurance (QA). Lot history files should be constructed for each ancillary material.▲ (USP 1-May-2020)

Conformance to ▲▲ (USP 1-May-2020) specifications should be compared to ▲▲ (USP 1-May-2020) the certificate of analysis ▲data.▲ (USP 1-May-2020) Traceability is essential, and lot numbers for each ancillary material used should be noted in the productions records of the cell-based product. ▲Chapter ⟨1043⟩ contains▲ (USP 1-May-2020) specific information about implementing an appropriate qualification program for these materials. Other *USP* chapters provide considerations about the qualification of specific ancillary materials (e.g., *Bovine Serum* ⟨1024⟩, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩, and *Growth Factors and Cytokines Used in Cell Manufacturing* ⟨92⟩).

Qualification of Excipients

During the final steps in the manufacturing process, excipients or substances that increase stability ▲and functionality▲ (USP 1-May-2020) of the therapeutic cells may be included. Examples of excipients include culture media, saline TS or other electrolyte solutions approved for injection, exogenous proteins such as human serum albumin, or cryoprotectants such as dimethyl sulfoxide (DMSO). Excipients are not intended to exert a direct therapeutic effect upon the patient; rather they are intended to contribute to the maintenance of the quality attributes of the final cellular product. Because excipients will be administered to the patient along with the cells, particular attention must be paid to their qualification. In general, excipients that are already FDA-approved for human use should be used whenever possible. If non-approved excipients are used, a complete safety assessment should be done. For novel excipients such as cryopreservation solutions, appropriately designed preclinical safety studies may be needed. ▲In addition, preclinical studies should include product formulated the same way as intended for clinical use.▲ (USP 1-May-2020)

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MANUFACTURING OF CELL▲-BASED ADVANCED THERAPIES▲ (USP 1-MAY-2020) OR TISSUE-BASED PRODUCTS

Introduction

The manufacturing of ▲cell-based advanced therapies▲ (USP 1-May-2020) or tissue-based products requires a number of operations and manipulations by individuals who are well trained in aseptic processing techniques. The technical competence of the personnel is particularly crucial to product safety and efficacy. ▲▲ (USP 1-May-2020)

Cell Isolation and Selection

▲Regardless of the source, there exist several generally-accepted principles for processing human or animal cells and tissues. The source cell or tissue material should be packaged in sterile, leak-proof containers and transported from the procurement area to the processing area under controlled conditions optimized to maintain quality. The transport medium may contain antibiotics, but care should be taken during processing so that antibiotics are not present in the final cellular product. In the case of blood-derived materials, or tissues containing substantial amounts of blood, the transport medium should contain an anticoagulant.▲ (USP 1-May-2020)

ISOLATION

▲Solid organs or tissues are usually dissected to expose a desired region. If multicellular organoids (for instance, islets of Langerhans) or single-cell suspensions are desired, the tissue may be subjected to mechanical or enzymatic disaggregation. In order to prevent excessive cell clumping, enzymes such as deoxyribonuclease may be added to digest nucleic acids released from damaged cells.

Cell and tissue isolations involving open manipulation steps should be carried out in an ISO 5 (Class 100) biological safety cabinet; the environment surrounding the biological safety cabinet should maintain aseptic processing operations. For minimally manipulated HCT/Ps in closed systems, these environments may be controlled but unclassified. However, for cell and tissue-based therapies that are manipulated and manufactured under cGMPs, the environment surrounding the biological safety cabinet should be controlled and classified, usually as an ISO 7 (Class 10,000) clean room. In all cases, proper in-process labeling, line clearance, and lot segregation should be employed in order to avoid product cross-contamination.▲ (USP 1-May-2020)

SELECTION

▲Cell mixtures may require further processing in order to enrich a desired cell population or remove an undesirable cell type.

Cell populations can be enriched by varying the force and duration of centrifugation, often in conjunction with a variable density gradient medium. Continuous-flow elutriation centrifuges separate cell populations by subjecting them to opposing centrifugal and fluid stream forces within a special rotor chamber.

Cell separation can also be achieved by taking advantage of cytological or biochemical characteristics unique to different cell populations. Soybean agglutinin aggregates cells that bear a particular carbohydrate moiety expressed on mature blood cells, but not stem cells, allowing enrichment of the stem cells. Certain cell populations adhere to specific solid substrates such as tissue-culture plastic, collagen-coated materials, or polymeric scaffolds; this allows the surface-bound cell type to be selectively removed from the initial cell suspension.

Monoclonal antibodies directed against specific cell-surface proteins can be used for positive or negative cell selection. For example, a monoclonal antibody-bound cell population can be removed from the cell suspension after incubation with antibody-specific magnetic nanoparticles, followed by magnetic collection. Target cell populations can be isolated from unlabeled cell suspensions by incubating the suspension with antibody-coated plastic surfaces or microspheres. A fluorescence-activated cell sorter (FACS) can be used to separate different cell types bound to fluorescently-labeled antibodies.

Cell populations may also be enriched by destroying unwanted cells, e.g., via antibody binding and exogenous complement fixation, or by incubation with cytotoxic agents or mitotic inhibitors. Such destructive procedures require thorough processing to the remove dead cells, cell fragments, and cytotoxic agents from the final cell product.▲ (USP 1-May-2020)

Cell Ex Vivo Expansion and Differentiation

EX VIVO EXPANSION

A key issue for manufacturers of ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue-based products is the ability to produce and deliver a therapeutically relevant dose of the required cell population to the patient. Depending on the application, the product may be a pure, homogeneous cell type, or it may be a mixture of different functional cell types. Many target cell populations are present at a low level or low purity in complex primary source tissues. In such cases, production of a therapeutic dose ▲are typically achieved▲ (USP 1-May-2020) by specific enrichment and ex vivo expansion of the required cells.

Ex vivo expansion of cells may occur in suspension culture (e.g., T cells or hematopoietic stem and progenitor cells), adherent culture (e.g., mesenchymal ▲stromal▲ (USP 1-May-2020) cells, embryonic stem cells, induced pluripotent stem cells, neuronal stem cells, or dermal fibroblasts), or a mixture of both (e.g., bone marrow stroma expansion). Numerous technologies exist for cell culture. Cells can be propagated in tissue-culture flasks (T flasks), in roller bottles, on polymeric scaffolds, or in nonrigid,

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gas-permeable bags, usually inside incubator units controlled for temperature, humidity, and gas composition. Multilayered, high-capacity cell culture systems composed of tissue culture plastic, multibag systems, and bioreactors using microcarriers enable expansion, harvesting, and formulation to be carried out in a closed system. Traditional small-scale fermenter units can be used for expansion of cells in suspension culture. It is also possible to expand adherent cells in such units either by providing a surface for attachment (microcarriers, coated beads, or disks) or by adapting the cells to propagate in suspension culture. Some culture systems are specifically designed for the propagation of cells for therapeutic applications. These tend to be closed systems that use disposable bioreactor cartridges in automated processing units with direct control of temperature, gas composition, and media perfusion rate. In some cases automated software allows patient–donor tracking and documentation of culture conditions and manipulations. These features are useful in the design and implementation of quality control (QC) product release testing programs and for the QA documentation of processing runs.

In adherent culture, the cells are usually harvested from the surface upon which they have expanded. Methods of release include physical agitation, enzymatic cleavage, and chelation of metal ions and competitive inhibition of adhesion or matrix molecules. As described above, consideration must be given to the source, safety, toxicology, and residual testing for any reagent used to release adherent cells during manufacturing. Some product-specific systems do not require the release of adherent cells. Cells are expanded on a biocompatible synthetic or natural scaffold that is then applied topically (for example, engineered skin substitutes), or the cells are grown inside or outside of fibers for ex vivo perfusion (for example, hepatocytes in hollow-fiber devices to treat liver disease).

In all cases standard cell culture parameters should be optimized for maximum process efficiency ▲and consistency.▲ (USP 1-May-2020) Such parameters include composition of cellular source material, initial seeding density, media composition, rate of media exchange, temperature, gas composition, pH, and rate of delivery. Depending on the nature of the product, the potential effect of process parameters on the potency and function of the target cells should be defined.

Bioreactors: Specialized bioreactors and devices ▲may be needed▲ (USP 1-May-2020) for manufacturing ▲certain▲ (USP 1-May-2020) three-dimensional combination products. These bioreactors hold the biocompatible scaffold/matrices for the manufacture of the construct. Although the bioreactor can provide a closed system for construct manufacturing, it creates a challenge in providing access to the scaffold for seeding cells and sampling for product release testing while maintaining sterility. Bioreactors are often single-use devices that ensure that no cross-contamination occurs between products. Preferably the product will not be repackaged for transport and delivery. For example, bioreactors may also serve as the final container for product shipment.

Container–closure testing must be performed for all final container–closure systems. Compatibility for sterilization of the bioreactor and the scaffold should be verified, and the sterilization process must be validated for each product configuration. Leachables and extractables from product-contact materials such as bioreactors and packaging components should be quantified, and limits should be established.

In closed bioreactor systems it can be difficult to observe or sample cells. Measurement of metabolic parameters can provide a surrogate method that is amenable to validation with which to evaluate the rate of proliferation and predict when to harvest the cell product. The relationship of such parameters to the viability, potency, and function of the cell product should be well defined. Postexpansion purification and enrichment of target cells by using methods such as those described above may be required.

DIFFERENTIATION

Some cell therapies require lineage or functional differentiation of the source cells. For example, hematopoietic stem cell expansion processes normally result in products that contain a mixture of multipotent stem cells, lineage-committed progenitor cells, and lineage-differentiated cells. The composition of these products can be manipulated by different combinations of growth factors and cytokines during the expansion process. The inverse is true for processes in which mature cells are "de-differentiated" to enable them to then be recommitted to a lineage pathway (for example, chondrocytes in cartilage repair). Specific examples of ex vivo manipulation are the production of antigen-specific T cells to target various specific disease indications or derivation of therapeutic cell types from embryonic stem cells. Before release for clinical use, the resulting differentiated target cells ▲should be appropriately▲ (USP 1-May-2020) characterized. Assessing the potential for de-differentiation of multipotent cells that have undergone differentiation may be necessary to ensure the safety of the product. Where the cells have been expanded and subsequently differentiated, karyotype analysis or in vitro transformation assays may be performed to demonstrate the cells are acceptable for clinical use.

EX VIVO GENETIC MANIPULATION

Genetic modification of cells ex vivo is a common processing procedure that is used to alter the pattern of gene expression in a defined population. The introduction of integrating or nonintegrating genetic materials (DNA, RNA, siRNA, ▲virus)▲ (USP 1-May-2020) is performed in order to induce the expression of new genes and products or to ▲change▲ (USP 1-May-2020) endogenous gene expression. Ex vivo genetic modification in autologous transplantation settings involves the manipulation of a harvested or expanded cell population from a patient and subsequent re-administration of the cells to the donor. In a typical allogeneic transplant setting, a stable, genetically modified cell population that has been characterized and banked is administered to a broad patient population. In order to control graft-versus-host disease in allogeneic bone marrow transplants, selected donor T cells have been treated with lethal genes such as thymidine kinase that make the cells susceptible to gancyclovir treatment after transplant. Examples of autologous genetically modified cell therapy products include ▲chimeric antigen receptor T-cells (CAR-T),▲ (USP 1-May-2020) the transduction of tumor cells with cytokine or other immunomodulatory genes, lymphocytes transduced with receptors for tumor antigens, and the introduction into harvested lymphocytes of an antiviral ribozyme vector as a strategy to treat human immunodeficiency virus infection. Allogeneic cell therapy product examples include ▲CAR-T,▲ (USP 1-May-2020) genetically modified and irradiated tumor cell lines used as tumor vaccines, and encapsulated cells transfected with a gene to express a neurotrophic factor for localized therapeutic protein delivery in the central nervous system.

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Ex vivo genetically modified cells are considered gene therapy. Issues associated with gene therapy products are addressed in detail in ⟨1047⟩, especially the production of the vector or genetic material used to accomplish gene transfer, analytical testing strategies, patient safety, and monitoring. The manufacturing, cell processing, and process control methodologies addressed above are applicable in the procedures used for genetic manipulation. Frequently cell populations that are genetically modified are isolated and▲/or▲ (USP 1-May-2020) expanded or selected before the introduction of the genetic material. Specialized equipment and processes for introduction of genetic material ▲must▲ (USP 1-May-2020) be validated and monitored. Issues associated with cell banking and stability apply to cell lines used in ▲cell therapy product manufacturing.▲ (USP 1-May-2020) Finally, issues associated with analysis and administration of the genetically modified cell population are discussed later in this chapter.

Formulation of Cell and Tissue-Based Products

Approaches for formulating cell and tissue-based products depend largely on the planned storage time for the cells before delivery to the patient. For some cell-based products, the time between completion of manufacturing and delivery to the intended recipient can be measured on the order of hours to days. Other cell-based products may be cryopreserved in order to extend their shelf life. A different approach for formulating cell and tissue-based products may involve the addition of a natural or synthetic scaffold that can facilitate handling, protecting the cells from immunological responses, and creating a specific shape that contributes to the therapeutic effect. Considerations for formulating each of these types of cell and tissue-based products are discussed below.

▲Decellularized Tissue-Based Products

The cells from decellularized tissue materials are killed, lysed, and removed from tissue using one or more of a variety of methods. Protocols may include physical methods (e.g., exposure to freezing temperature, force and pressure, or electrical disruption) and/or exposure to enzymes or chemicals (e.g., acids, bases, or detergents). These step(s) are typically followed by thorough rinsing and soaking in buffer to remove reagents and all cellular debris, as well as to neutralize reagents as needed. Tissues may then be further processed, such as demineralization (bone), before packaging, freeze-drying, and/or other final processing steps.▲ (USP 1-May-2020)

NONCRYOPRESERVED CELL ▲▲ (USP 1-May-2020)-BASED PRODUCTS

Products consisting of suspensions of cells for delivery to patients within hours after the completion of manufacturing frequently are formulated in sterile, buffered solutions suitable for direct administration. For other noncryopreserved cell ▲▲ (USP 1-May-2020)-based products extension of shelf life from hours to days may be possible by use of solutions that contain appropriate nutrients and antioxidants. In most cases, these excipients are not intended for direct administration into patients. Consequently, the excipients may require removal before delivery to the patient (see *Clinical Site Preparation and Administration*). If an unapproved formulation buffer will be administered to patients, preclinical toxicology testing should be performed.

CRYOPRESERVED CELL ▲▲ (USP 1-May-2020)-BASED PRODUCTS

Most cell cryopreservation medium formulations are supplemented with 5%–10% DMSO with or without hydroxyethyl starch (generally 6%) and a plasma protein such as 4%–10% human serum albumin in a balanced salt solution. DMSO prevents dehydration by altering the increased concentration of nonpenetrating extracellular solutions during ice formation. The high molecular weight polymeric hydroxyethyl solution protects the cells from dehydration as water is incorporated into extracellular ice crystals. The use of protein often results in maximum recovery and viability of cells after thawing. ▲▲ (USP 1-May-2020) Some cryopreservation formulations are completely free of protein.

The optimal concentration of cells for cryopreservation depends on the cell type and should be determined empirically, but it generally ranges from 10^6 to 10^7 cells/mL. The homogeneity and viability of the cell population being cryopreserved can also differ after thawing and should be carefully assessed. In situations where the final ▲cell-based▲ (USP 1-May-2020) product is intended to be thawed and administered immediately, the presence of DMSO in the formulation buffer does subject the patient to an increased level of infusion-related toxicity, although this is related to the volume administered and the final concentration of the cryopreservative. Refer to *Clinical Site Preparation and Administration* for additional considerations.

CELLS COMBINED WITH BIOCOMPATIBLE SCAFFOLDS

Many cell and tissue-therapy products are administered in combination with a biocompatible scaffold ▲(also referred to as tissue engineering). Some▲ (USP 1-May-2020) wound healing or skin substitute products contain cells seeded on a scaffold. The biochemical and physical structure of the scaffold and the method for combining cells with the scaffold are specific to the product. ▲The biocompatible scaffold (e.g., demineralized cortical bone particles, cancellous granules) may also be human tissue derived from the same donor as the allogeneic cells.▲ (USP 1-May-2020)

Cells can be loaded into a semipermeable membrane device for delivery. Usually the pore size of the membrane is large enough to allow the cell-secreted therapeutic factors to pass, but it is small enough to stop immunoglobulins and host cells from making contact with, destroying, or mounting an immune response to the therapeutic cells. The device can be a single hollow-fiber or a semipermeable capsule with cells inside that secrete therapeutic compounds, or it can be part of a larger system of pumps and filters such as hollow-fiber modules with hepatocytes for the treatment of liver disease.

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Cells can be seeded onto a three-dimensional scaffold and allowed to propagate and form a tissue-like structure. In the resulting product, the cells are oriented in a unique manner that is important for the intended use of the product (e.g., skin substitutes).

Cells can be encapsulated in a gel or cross-linkable polymer solution, and the resulting implantable structure can serve as a culture vessel, as a means to shield the cells from the host's immune system, or as a way to mold cells into a defined shape. Some of the polymers used include alginate, hyaluronic acid, collagen, chitin, or synthetic polymers. Encapsulated pancreatic β-islet cells have been implanted in patients to treat diabetes. To treat urinary incontinence, chondrocytes have been mixed with alginate to form a structure upon injection.

Cells can be adhered to scaffolds of defined shape that are then implanted. Some examples include osteogenic precursor cells on scaffolds of demineralized cadaveric human bone, ceramic hydroxyapatite, ceramic hydroxyapatite–tricalcium phosphate, or biodegradable glass, which can be used in the repair of bone defects.

Change to read:

ANALYTICAL METHODS

General Considerations

The complexity and scope of cell-based therapies are reflected in the wide range of analytical methods that are used to establish in-process controls and final product release criteria. Quality specifications for ▲cell-based advanced therapies▲ (USP 1-May-2020) and tissue products should be chosen to confirm the product's quality, safety, and potency. Selected tests should be product specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and tissue products should follow the principles outlined in ICH Q6B, *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*.

Specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Manufacturers should develop specifications for each product developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies, and should also include assay and process validation data that can be correlated to stability, safety, and efficacy assessments.

In-process controls and specifications for the product should ▲ideally▲ (USP 1-May-2020) be anchored by use of an appropriate reference standard ▲or reference material.▲ (USP 1-May-2020) An autologous product may rely on a reference standard generated from processing cells or tissue from a healthy donor or from a source that supplies cells and tissues to research institutions. The reference standard ensures that the process, as measured by the release assays, does not change significantly over time, and it verifies that a test produces acceptable results, i.e., system suitability requirements are met. The reference standard is made from a lot that is produced under controlled conditions and passes all in-process and final release testing. In addition, this reference standard is subjected to an additional level of characterization that includes tests not normally performed for product release. The reference standard need not be stored at the same dose, formulation, or temperature as the final product. However, the stability of this reference standard must be determined.

Alternatively, a working standard can be used. If so, in the test it should behave like the reference standard. Changing to a new reference standard should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted. One option for a reference standard for a cell product with a short shelf life or for an autologous or patient-specific application can be a bank of normal donor cells of the appropriate cell type. This cell bank can be used to ensure that the manufacturing process is capable of making a consistent product.

In-Process Controls

Manufacturing processes should have well-defined go–no go decision criteria that are established for key in-process manufacturing steps. In-process control ▲tests▲ (USP 1-May-2020) ensure that the in-process material is of sufficient quality and quantity to ensure manufacture of an acceptable final product. Examples of in-process controls include:

- ▲Cell▲ (USP 1-May-2020) enumeration and viability
- Microbiological (sterility, endotoxin, mycoplasma)
- Expression of phenotypic or genotypic markers
- Verification of morphology against visual reference standards
- Production of a desired bioactive substance
- Determination of population doublings, passage number, age of culture
- Assays ▲for▲ (USP 1-May-2020) process impurities
- Monitoring of culture system parameters [% carbon dioxide (CO_2), % relative humidity, pH, glucose, lactate, etc.]
- Functional tests such as colony forming units (cfu) and expression of cell-specific proteins
- ▲Content uniformity
- Quantitation of particulate matter
- Visual inspection for cellular aggregates and other impurities

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A primary reason for in-process control tests is to reliably obtain adequate product quality and yield. A secondary reason▲ (USP 1-May-2020) is to gather process and product characterization data that can be useful in assessing the impact of process changes or excursions. ▲Refer to *Risk Assessment* for discussion on critical process parameters (CPP). In-process sampling must always be balanced against adequate final product yield. Therefore, only truly informative and timely in-process testing should be implemented.▲ (USP 1-May-2020) Intermediate ▲▲ (USP 1-May-2020) process material that fails to satisfy in-process control criteria should not be used for further manufacturing. ▲Non-conforming▲ (USP 1-May-2020) material ▲may▲ (USP 1-May-2020) be reprocessed if there are procedures in place. ▲▲ (USP 1-May-2020) The effect of reprocessing on other quality attributes such as stability must be defined before the material can undergo further manufacturing. ▲The reprocessed material must satisfy the original in-process specifications.▲ (USP 1-May-2020) If several sub-lots (e.g., cells harvested from different culture vessels) will be pooled for further processing, ▲non-conforming sub-lots▲ (USP 1-May-2020) should not be included ▲▲ (USP 1-May-2020) even if the ▲larger▲ (USP 1-May-2020) pool containing these failed sub-lots would pass the in-process assay criteria. ▲For manufacturing processes that yield multiple containers of cell suspensions, in-process visual inspections are highly recommended. For example, fibers, polymer fragments and other visible particulate matter can accumulate and co-mingle with cells presenting a patient safety concern. In-process visual inspection after the final container filling, but prior to the final product labeling or cryopreservation (if frozen), allows the culling of nonconforming containers before final product sampling and testing. Timely in-process visual inspection may be challenging, particularly when attempting to limit cell exposure to cryopreservatives such as DMSO.[1] ▲ (USP 1-May-2020)

During ▲▲ (USP 1-May-2020) process development, assays for product quality and yield should be performed after most processing steps to determine which steps are ▲quality-critical, stability-indicating, or sensitive to process deviations. Statistical process controls and critical product quality parameters, along with statistically valid sampling, should be used to establish limits for process validations and manufacturing investigations. In-process controls should be performed even for fully validated manufacturing processes to ensure proper process control. Assay results should be tracked and trended over time, and actions should be taken to correct problems as they arise.▲ (USP 1-May-2020)

Final Product Release Specifications

Cell-based therapies regulated as biological products must comply with applicable sections of 21 CFR 211 and 21 CFR 610 to ensure ▲they meet essential quality attributes such as▲ (USP 1-May-2020) identity, purity, potency, and microbiological safety. ▲▲ (USP 1-May-2020)

Because terminal sterilization is not possible for a living cell-based product, essentially all cell-based products are required to meet acceptance criteria for product tests such as sterility, mycoplasma ▲(not needed if cells are cultured less than 24 h, or if tissue has been decellularized),▲ (USP 1-May-2020) and endotoxin—typically, negative or no growth demonstrates sterility and the absence of mycoplasma, and products must demonstrate <5 endotoxin units (EU) per kilogram of patient body weight. In the case of intrathecal injection, the specified endotoxin limit is more stringent: ≤0.2 EU/kg of patient body weight. Adventitious virus testing is rarely performed on the final cell-based therapy product because the source cells or cell banks and ancillary materials of biological origin have been screened and tested for viral agents of concern before manufacturing.

For almost all other final product release criteria, such as those for identity, purity, and potency, the analytical procedures with methods and acceptance criteria are specific to the individual cell-based product. *Table 3* provides an overview of the expected final product release tests for cell-based therapies and lists examples of approaches that are used to satisfy the testing requirements.

Table 3. Overview of Final Product Release Testing

Release Test	Examples	Criteria
Sterility	*Sterility Tests* ⟨71⟩	Negative
Mycoplasma	Direct and indirect culture method (FDA *Points to Consider*)	Negative; not detected. ▲Testing not usually required if cells are cultured less than 24 h.▲ (USP 1-May-2020)
Endotoxin	*Bacterial Endotoxins Test* ⟨85⟩	<5 EU/kg (<0.2 EU/kg intrathecal)
Identity	• Surface marker determination • Isoenzyme analysis • Genetic fingerprint • Morphology • Bioassay • Biochemical marker	Product specific
Purity	• Percentage of viable cells • Percentage of cells expressing specific marker(s) • Limits on undesired cell types • Limits on process contaminants (e.g., serum) • ▲Scaffold composition▲ (USP 1-May-2020)	Product specific

[1] Clarke D, Jean S, Powers D, Karnieli O, Nahum S, Abraham E, et al. Managing particulates in cell therapy: guidance for best practice. *Cytotherapy.* 2016;18(9):1063–1076.

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Table 3. Overview of Final Product Release Testing *(continued)*

Release Test	Examples	Criteria
Potency	• Viable cell number • Colony-formation assay • Change in expression of specific genes • Secretion of desired macromolecule • ▲Expression of cell-surface molecules▲ (USP 1-May-2020) HLA • ▲Target specific cell killing▲ (USP 1-May-2020) • Evidence of metabolic activity • Evidence of cell function	Product specific
Dose	• Viable cell number • Enumeration of specific cell population • Total DNA • Total protein	Product specific
▲Appearance	*Injections* ⟨1⟩, *Visible Particulates in Injections* ⟨790⟩	Product specific. Cell suspensions should be free of unexpected visible particulate matter.▲ (USP 1-May-2020)
Others	• ▲▲ (USP 1-May-2020) • Morphology • Size • Tensile or burst strength	Product specific

STERILITY

Cell ▲and tissue▲ (USP 1-May-2020)-based products are required to comply with final product release testing requirements, including sterility. Sterility testing is also frequently performed in-process to establish microbial purity for cells that require extended culturing. A suitable sterility test includes the test described in ▲▲ (USP 1-May-2020)*Sterility Tests* ⟨71⟩. These culture-based test methods require 14 days and thus are suitable only for cell-based therapy products that have extended shelf lives (e.g., following cryopreservation). Many cell-based therapies have short shelf lives and must be delivered to patients before the 14-day test results are available. In such situations, the FDA has identified an approach that will allow the administration of the cell-based product to patients in this setting [see *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*]:

- In-process sterility testing on a sample taken 48–72 h before final harvest or after the last refeeding of the cultures
- A rapid microbial detection test such as a Gram stain or other procedure on the final formulated product
- Sterility testing compliant with 21 CFR 610.12 on the final formulated product

Under this alternative approach, the release criteria for sterility would be based on a negative result of the Gram stain and a no-growth result from the 48- to 72-h in-process sterility test. In the event that the 14-day sterility test is determined to be positive after the product is administered to the subject, the manufacturer is required to report the sterility failure, results of investigation of the cause, and any corrective actions as an amendment to the IND within 30 calendar days after initial receipt of the positive culture test result.

Because of concerns regarding the sensitivity of a Gram stain and the inability to obtain full sterility results for 14 days after administration to the patient, there is widespread interest in the use of rapid microbiological methods as an alternative to the 14-day culture method. This is discussed in *Alternative Test Methodologies*.

MYCOPLASMA

▲▲ (USP 1-May-2020)

Testing for mycoplasma is recommended for all raw materials derived from a human or animal source; ▲it▲ (USP 1-May-2020) is required as a lot-release assay for cell-▲and tissue-based▲ (USP 1-May-2020) products ▲if they are cultured for longer than 24 h.▲ (USP 1-May-2020) The FDA has published a document (*Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*) describing in detail the accepted methods for the cultivation and isolation of mycoplasma. Methods for mycoplasma detection are also described in *Mycoplasma Tests* ⟨63⟩. Because the classical assay takes 1 month ▲▲ (USP 1-May-2020) to complete, alternative methods are being developed and validated for the rapid detection of mycoplasma; this is discussed in *Alternative Test Methodologies*.

ENDOTOXINS

▲Endotoxins exert numerous biological effects: they can affect secretion and cytokine production, induce fever in recipients, or serve as powerful mitogens. Because of these wide-ranging impacts, endotoxin assessment must be included in raw material and manufacturing process qualifications for cell and tissue-based therapies. *Bacterial Endotoxins Test* ⟨85⟩ describes methods for measuring endotoxins, all based on the Limulus amoebocyte lysate assay. This assay can be validated for a wide range of biopharmaceutical products; one important feature is the ability to rapidly conduct the assay before the release of short-shelf-life products.▲ (USP 1-May-2020)

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IDENTITY

▲Lot-release testing for cell-based products must include an identity test to unequivocally identify the product. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured, e.g., in a single-product vs. a multi-product facility.

Identity tests for cell-based products must be relevant to the cell type and manipulations applied during processing. Differential surface markers are frequently used to ascertain product identity, as described in *Table 3*. Flow-cytometric immunoassay methods are the most common means of detecting and quantifying these markers. Isoenzyme analyses can confirm species of origin, which is especially useful for xenogeneic cell products. Cell morphology may be used to distinguish specific cell types. Genetic fingerprint technologies such as short tandem repeats can also establish cell line identity.▲ (USP 1-May-2020)

PURITY

▲Purity methods specifically quantify the intended active product components, or unwanted impurities (product- or process-related residual contaminants), in the final product. *Table 3* describes examples of several impurity methods. The requirement to test for a particular impurity for product lot release depends on: 1) the capability of the manufacturing process to remove or inactivate the impurity, as demonstrated by process validation, and 2) the impact of the impurity, such as toxicity or altered product function.▲ (USP 1-May-2020)

Examples of process-related impurities associated with ▲cell-based▲ (USP 1-May-2020) therapy products include residual production-medium components (e.g., serum, antibiotics, or exogenous cytokines), ancillary materials used in downstream processing (e.g., nucleases or proteases), and leachables (e.g., plasticizers from tubing or culture plastic). Impurities may be bioactive (e.g., cytokines or hormones) or immunogenic (e.g., aggregates, degradation products, or animal-derived proteins). Impurities may have other deleterious effects, depending on the dose of the product.

▲Product-related impurities are specific to each product type. Impurity examples include cell debris; cells that are dead, non-functional, altered, or otherwise unwanted. Analytical methods should quantitate both product and impurities, and should allow the manufacturer to assess the lot-to-lot consistency of the product-impurity profile. It may be possible to validate the manufacturing process to the extent that specific lot-release testing for impurities can be limited.

Testing for impurities is often extensive during product characterization and process validation, when the consistency of the manufacturing and purification process is being assessed. In-process testing for impurities can provide valuable information regarding the consistency of manufacturing and materials. *In-Process Controls* describes the role that in-process testing can play in monitoring and limiting impurities, both during process development and manufacturing. Lot-release impurity testing should reflect the safety risks associated with the impurity, and the ability of the process to consistently remove that impurity.▲ (USP 1-May-2020)

POTENCY

Potency is defined under 21 CFR 600.3(s) as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*, below). ▲The relationship between product potency measurements and clinical safety and efficacy is key to their use in batch release. Potency may be assessed by cell enumeration, in vitro or in vivo bioassays, or a combination of these. Because it is not uncommon for potency assays to have large coefficients of variation, these assays often require well-defined and fully characterized representative reference material. In part because of their inherent variability, patient-specific products such as autologous immunotherapies present a particular challenge in demonstrating potency. Potency assay development should focus on characterizing and controlling the sources of assay variability. Information about potency assay and product variability should inform the product stability study design, as well as the proposed statistical approach to expiration date assignment▲ (USP 1-May-2020) (see *Stability*, below).

▲Assays measuring the potency of cell-based therapy products vary widely, and depend on the products' unique characteristics and shelf life. For some cell-based products, product potency may correlate with clinical efficacy. For example, a colony-forming assay or enumeration of committed progenitor cells may correlate with clinical engraftment outcomes in clinical studies. Product potency may also correlate with responses measured in animal or cellular disease models. If the cell-based product releases a bioactive macromolecule, a potency assay could be based on the units of activity released. For example, the production of insulin in response to changes in glucose levels could be the basis of a potency assay for a cell-based therapy product intended to treat diabetes.

Approaches to measuring product potency should be discussed with regulatory authorities early in development. The FDA has issued guidance that discusses using a matrix of biological and nonbiological assays, including both qualitative and quantitative assays, to establish product potency. Information in this guidance is particularly relevant for cell-based products that have a short shelf life, a complex mechanism of action, or multiple biological activities (see the FDA's *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*).

A validated potency assay is typically required before regulatory approval, which means that a well-defined product potency assay (or assays) should be in place before the initiation of pivotal clinical trials. Therefore, implementation of one or more candidate potency assays early in clinical development is strongly encouraged. Data from these candidate potency assays can also be particularly useful for assessing proposed manufacturing changes, during technology transfer, and in determining product stability.▲ (USP 1-May-2020)

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DOSE-DEFINING ASSAYS

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision.

▲Cell-based▲ (USP 1-May-2020) therapy products may be dosed on the basis of enumeration of one or more cell populations. For products ▲consisting▲ (USP 1-May-2020) of a homogeneous, single-cell suspension, viable cell number is the most frequently used assay. Such assays may include enumeration of all cells, total nucleated cells, or another subset of cells. Viability assays are usually based on a cell's ability to exclude a supravital dye, such as trypan blue. Results are expressed as the number of cells that exclude the dye and are therefore considered viable. Fluorescent compounds that bind to nuclear proteins and are excluded by viable cells may be incorporated into flow-cytometric methods for simultaneous determination of viability and cell-identity markers.

Cell counting may be performed rapidly by manual or automated methods. Manual cell counting by visual enumeration of cells in a hemacytometer chamber is a readily available technique with acceptable accuracy but a lower degree of precision than most automated methods. Typical instruments for automated ▲blood▲ (USP 1-May-2020) cell counting provide reproducible enumeration of nonnucleated cells (e.g., erythrocytes and platelets) and nucleated cells and differential counting of the nucleated cells into mononuclear and polymorphonuclear leukocyte populations. ▲Other automated cell counters relay on computer algorithms for visual field analysis of stained cell populations. Beyond cell counting, further identification and enumeration▲ (USP 1-May-2020) of specific cell populations usually requires cell-surface phenotype analysis by flow cytometric or other methods (see *Identity* above). The proportion of a specific subpopulation of cells may be determined by FACS analysis or by flow cytometry. ▲An example of a specific cell enumeration assay that incorporates viability assessment is described in *Flow Cytometric Enumeration of CD34+ Cells* ⟨127⟩.▲ (USP 1-May-2020)

For products that contain cells in a nonhomogeneous suspension, such as cells that are combined with a biomaterial (e.g., a scaffold), alternative cell enumeration measures have been used, including total area of a cell sheet, wet weight, total protein, and total DNA. If such measures are used to determine product dose, then supplemental tests should be performed to establish relevance.

Considerations for Release Testing of Cell–Biomaterial Constructs

For some cell-based ▲therapy▲ (USP 1-May-2020) products such as cells combined with biomaterials to form combination products, it may not be feasible to directly test the cell–biomaterial construct. This is frequently the case when autologous cells are involved and the cell–biomaterial construct consists of a single unit and sampling of the construct is not feasible. In such cases, the individual components are tested before they are combined, and the final construct is not subjected to direct testing. Indirect measures such as sampling of the culture media can be employed to address regulatory requirements. The quality and stability of the formulated cell–biomaterial construct and relevance of indirect measures must be established by validation studies during product development.

Sampling Issues

▲GMP samples from each lot must be retained after the completion of final product release testing, in case safety or quality issues arise later. Even if the product has a very short shelf life, retained samples can be used to detect impurities and other unwanted substances. If rapid-release strategies are employed, manufacturers may need to retain additional or alternatively stored (such as frozen or fixed) samples, so that product quality can be reassessed if necessary. In all cases, sample handling and storage must be validated against relevant testing methods and stability requirements. The FDA (in 21 CFR 820.250) requires product and material sampling plans to be written, specific, and justified on the basis of statistics, science, and risk. Sampling considerations are especially important for products dispensed into multiple containers. Sampling should be based on the appropriateness, stability, and expected distribution for each parameter tested. Proper sampling plan design should include considerations for samples (e.g., uniformity, size, handling, storage) and statistics (e.g., frequency, redundancy, confidence limits, replicates). Process development studies generally help determine the appropriate parameters and limits for the statistical sampling plan design; process validations must confirm or support those statistical sampling plan parameters and limits. See *Stability Protocol Development* (below) for additional considerations, and ⟨71⟩ or *Visible Particulates in Injections* ⟨790⟩ for examples of statistical sampling approaches.▲ (USP 1-May-2020)

Alternative Test Methodologies

As described in *Final Product Release Specifications* (above), cell-based therapies must undergo testing for sterility, mycoplasma, and endotoxin. Additional acceptance criteria for tests relating to identity, purity, potency, dose, and other relevant characteristics must be met before clinical use. With the exception of sterility, mycoplasma, and endotoxin, most of the test procedures and their underlying methods used to ensure that the final product meets release acceptance criteria are unique to the product and can be adapted to meet the specific characteristics and applications of the cell-based ▲therapy▲ (USP 1-May-2020) product. In general, test methods should be developed based on the best available science and should be suitable for use in a GMP manufacturing environment. The assays should be robust, reliable, and capable of being validated and should provide results before release for clinical use. *Validation of Compendial Procedures* ⟨1225⟩ provides basic considerations for methods validation.

For some cell-based therapies, the sample size and volume of material required for testing or the length of time necessary to obtain test results can consume significant amounts of the final product, or the time required for obtaining results may exceed the product's shelf life—or both. This creates problems with the available supply of product to treat patients and in other situations precludes the possibility of obtaining results before administration to patients. This is a particular problem for the

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compendial sterility test as well as the FDA-recommended broth–agar culture method for mycoplasma. Consequently, both industry and regulatory authorities have shown considerable interest in facilitating the development of alternative test methods for both sterility and mycoplasma.

FDA regulations for biological products specifically address the use of equivalent methods provided they ensure that the safety, purity, potency, and effectiveness of the biological product is equal to or greater than the assurances provided by the specified method (21 CFR 610.9). Some of the available alternative test methods for sterility and mycoplasma are described below.

The range of available technologies is broad and continues to be developed by assay designers for use in the cell therapy industry. Attributes that should be included in any review of proposed technology include accuracy for the intended purpose, speed in productivity, cost, acceptability by the scientific community and regulatory agencies, simplicity of operation, training requirements and reagents, the reputation of the vendor, technical services provided by the vendor, and, finally, utility and space requirements.

Validation of these test methods and demonstration of equivalence as described in 21 CFR 610.9 are required at the time of BLA or a PMA submission.

STERILITY

Detection platforms for alternative microbiological methods have been ▲described in *Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach* ⟨1071⟩.▲ (USP 1-May-2020) The primary advantage of these systems is the automated nature of the test results and recovery of microorganisms for failure investigations and other microbial characterization methodologies. ▲▲ (USP 1-May-2020) Principles of validation of alternative microbiological methods are also described in *Validation of Alternative Microbiological Methods* ⟨1223⟩.

▲

▲ (USP 1-May-2020)

MYCOPLASMA

Compendial testing methodologies for mycoplasma are growth based in agar and broth cultures and require at least 28 days to monitor appropriately the presence of mycoplasma contamination. Because of this limitation, a number of rapid mycoplasma testing technologies have been developed based on nucleic acid amplification techniques such as PCR, as well as nonamplified nucleic acid hybridization assays, enzyme-linked immunosorbent assays (ELISA), and enzyme-based assays.

Change to read:

QUALITY SYSTEMS

▲Quality systems weave together all aspects of manufacturing. As with biopharmaceutical and pharmaceutical products, the FDA requires manufacturers of cell-based therapies and tissue-based products to employ QC and QA programs to oversee manufacturing facilities, processes, validation efforts, document and change management systems, labeling, employees training, and the testing of all raw materials, in-process samples, bulk products, and final formulated products. Human cells and tissue must be handled in accordance with GTP as described in 21 CFR 1271. In addition, cGMP as outlined by the FDA in 21 CFR 210, 211, 600s (especially 21 CFR 610), and 820 apply to the manufacturing of cell-based therapies and tissue-based products that are subject to PMA. The extent of quality oversight increases as clinical development progresses, and full cGMP compliance is expected by the time Phase III clinical trial(s) begin.▲ (USP 1-May-2020)

Data obtained from in-process and final product release testing should be ▲recorded and▲ (USP 1-May-2020) monitored. Results that are out of specification (OOS), or even those that are out of trend, must be investigated before disposition of the material. The FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* (October 2006) provides a systematic approach for conducting an investigation.

▲▲ (USP 1-May-2020) An effective risk-management approach at the earliest stages of ▲cell-based▲ (USP 1-May-2020) product development ▲helps▲ (USP 1-May-2020) ensure the highest ▲product quality by proactively understanding,▲ (USP 1-May-2020) identifying, and mitigating potential quality issues. ▲▲ (USP 1-May-2020) Risk management can ▲also▲ (USP 1-May-2020) be used to establish meaningful specifications and ▲CPPs▲ (USP 1-May-2020) to ensure that quality attributes are met.

Risk analysis ▲and mitigation systems use▲ (USP 1-May-2020) experience and process knowledge to define risk categories. ▲▲ (USP 1-May-2020) As an example, Risk Levels 1–4 are defined below and can be adopted as one means of conducting a preliminary risk assessment:

Risk Level 1: *Technicality*—Poses no risk to the patient and does not impact the safety and effectiveness of the product. Example: A missing signature on a batch record.

Risk Level 2: *Alert*—May pose a safety risk to the patient or may have a potential impact on the safety and efficacy of the product. Compliance must be re-established with appropriate justification to proceed after ▲QA▲ (USP 1-May-2020) review and approval. Example: Digestion time for biopsy processing falls outside a defined range.

Risk Level 3: *Do not ship/reject lot*—May pose a safety risk to the patient or ▲may▲ (USP 1-May-2020) impair the efficacy of the product even after corrective action. Shipment is not permitted. Example: Cultures fail to demonstrate adequate cell growth.

Risk Level 4: *Post-Distribution Event*—May pose a safety risk to the patient or ▲may impair▲ (USP 1-May-2020) the safety, potency, or purity of the product. The ▲defect▲ (USP 1-May-2020) is identified after product distribution. Example: Failed sterility test occurred after distribution of product.

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Change to read:

FACILITY DESIGN AND OPERATION CONSIDERATIONS

▲Like biopharmaceutical operations, cell-based advanced therapy and tissue-based product▲ (USP 1-May-2020) manufacturing facilities ▲▲ (USP 1-May-2020) must be carefully designed to maintain ▲cGMP-compliant▲ (USP 1-May-2020) aseptic processing operations while also accommodating ▲unique product requirements.▲ (USP 1-May-2020) Cells or tissue ▲starting materials▲ (USP 1-May-2020) may need to be received and processed in a segregated area under quarantine to avoid ▲cross-contaminating▲ (USP 1-May-2020) the main facility. ▲Specially designed equipment and spaces may be required for tissue or cell processing. The facility may need to store, process, and dispose of specialized chemicals or infectious waste. All of these issues may constrain the design of the facility, especially air-handling systems and clean room environments.

While protecting the cell-based advanced therapy or tissue-based product from inadvertent contamination is a primary concern, minimizing risk to the manufacturing operators must also be ensured through appropriate training, protective clothing (gowns, gloves, sleeves, surgical masks, eye protection, and head coverings) and engineering controls (biological safety cabinets, appropriate air quality, etc.).▲ (USP 1-May-2020)

The degree of control required for cell and tissue processing operations depends on a number of factors, including the complexity of the aseptic manufacturing process, the primary site of manufacturing, and the final product shelf life. ▲▲ (USP 1-May-2020) ISO 7 (Class 10,000) clean rooms and ISO 5 (Class 100) biological safety cabinets are ▲typical▲ (USP 1-May-2020) components for cell therapy manufacturing processes, especially those that involve open manipulations.

▲▲ (USP 1-May-2020) The differential pressures between classified manufacturing should comply with the FDA's September 2004 document, *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice.* The facility and processing areas should be monitored for air quality in a manner that provides a high level of ▲aseptic assurances.▲ (USP 1-May-2020) For guidance in this area, see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩.

Facility cleaning, component and product segregation, sanitization procedures, ▲and environmental monitoring systems▲ (USP 1-May-2020) must be in place to avoid microbial contamination and cross-contamination between ▲production▲ (USP 1-May-2020) lots. ▲Techniques such as bar-coding and radio frequency (RF) tags can be used for product tracking and segregation. For guidance in this area, see 21 CFR 211.42, 211.113, 1271, and the FDA's September 2004 *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice.*

Unlike facilities designed for allogeneic products which rely on volume scale-up, facilities for autologous products typically utilize multiple small-unit scale out, which must be considered in the design and operation of the facility. Automation can be used to effectively manage repetitive manual cell manipulations and multiple simultaneous production lots.

Process equipment and software that controls or monitors critical parameters must be qualified and validated; such equipment should also be fitted with alarm systems and emergency back-up power.▲ (USP 1-May-2020)

Change to read:

CONSIDERATIONS FOR VALIDATION AND QUALIFICATION

The principles of validation recommended by ICH and FDA guidance documents and ⟨1225⟩ and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩ apply to cell or tissue-based products. ▲Analytical and manufacturing equipment and methods should be validated following the principles described in ⟨1225⟩, in addition to guidance documents issued by ICH [see Q2 (R1)].

Cell-based advanced therapies and tissue-based products present some unique validation challenges. First, the cellular or tissue starting materials can be quite variable, with a wide range in the quality and quantity. Second, manufacturing process steps (especially manual manipulations) can be complex and variable. Manufacturers should develop and validate raw material acceptance criteria, robust manufacturing processes, and analytical methods that consistently result in acceptable final products, even if the process relies on nonstandard or variable tissue materials.▲ (USP 1-May-2020)

Process validation should take this variability into consideration and should ensure that critical manufacturing and testing endpoints consistently meet specifications.

Aseptic process validations should be performed using ▲microbial growth media in place of the usual process materials, in order▲ (USP 1-May-2020) to show that the manufacturing staff can execute the procedures and produce a product free of microbial contamination. Procedures intended to maintain segregation during manufacturing should be challenged to verify that there is minimal opportunity for cross-contamination or ▲inadvertent exchange▲ (USP 1-May-2020) among different patient product lots. ▲Personnel training plans should be established, and proficiency qualifications should be performed periodically. At facilities where raw materials are procured or products are administered, personnel may need to be trained and qualified in order to minimize variability.▲ (USP 1-May-2020)

Depending on the variability in the source cells or tissues and the complexity of the manufacturing process, it may be necessary to manufacture more than three qualification lots to verify the consistency and the robustness of the manufacturing process. ▲Manufacturing data should be tracked and trended to enable manufacturers to discover and correct problems. Annual product reviews should summarize such factors as in-process and final product testing results, process failures, deviations, OOS, and effectiveness of corrective and preventive action (CAPA) over a 12-month period.▲ (USP 1-May-2020)

Equipment and facility cleaning validations should be performed to ▲evaluate cell and DNA carry-over, and to▲ (USP 1-May-2020) demonstrate the efficacy of cleaning ▲and sanitizing▲ (USP 1-May-2020) agents on ▲both▲ (USP 1-May-2020) standard microbial and fungal contaminants ▲and environmental isolates from the manufacturing facility. Measurement of residual cleaning agents should be addressed in equipment cleaning validations.

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Shipping validations, including environmental challenges such as extremes of temperature and vibration, should be performed for the cellular or tissue starting materials, as well as the final product. Validations should also be performed on software that tracks and controls labeling, shipping, and inventory systems. ▲ (USP 1-May-2020)

Change to read:

CLINICAL SITE PREPARATION AND ADMINISTRATION

▲Before administration of some cell or tissue-based products, one or more product modifications or preparative steps may be required. These modifications or steps are frequently performed close to the time of administration, and, therefore, they may not be under the direct control of the original manufacturer. The nature of these modifications is largely dictated by characteristics of the product.

At the clinical site, written procedures and process controls must be established for all product storage intervals, transport steps, and modifications, starting with a clear definition of critical control points. The unique and irreplaceable nature of many cell or tissue-based products heightens the need for well-established procedures for clinical site preparation and administration. ▲ (USP 1-May-2020)

Product Manipulations

▲ ▲ (USP 1-May-2020) Typical manipulations include the following:

- **Change in final container:** The manufactured product may have been stored or transported in one container and may require transfer to a different container for administration.
- **Change in physical state or temperature:** A product may require thawing or warming.
- **Change in solution or suspension:** A product may have to be dissolved, diluted, or suspended in a liquid.
- **Combination with a biomaterial:** Therapeutic cells may require combination with a scaffold material such as decellularized extracellular matrix sheets, gels, plugs, capsules, sponges, particles, or granules. In other cases, cells can be added to an existing medical device such as a hollow-fiber filtration unit before use.
- **Admixture or compounding:** For some cell products, mixing or compounding at the clinical site may be necessary.
- **Filtration or washing:** The presence of unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations, may require washing or filtration steps.
- **Sampling:** Sampling of the final product before administration may be required to test the final formulation.

▲ ▲ (USP 1-May-2020)

Thawing Cell-Based Products

Thawing is performed rapidly. If a small number of cells will be reinfused or transplanted, DMSO does not need to be removed from the suspension because most cell preparations can be concentrated adequately to keep the DMSO concentration within tolerable limits. DMSO use has two effects on cells after thawing: cells may clump if damaged, and DMSO reduces cell viability in minutes. If the DMSO must be removed or cells must be concentrated for administration, the thawed cell suspension is generally serially diluted (to avoid osmotic shock) and resuspended in a protein-containing medium. Cell viability and potency may be monitored after thawing, but the information is frequently intended only to gather information rather than as a specification that must be met for clinical use of the cellular product.

▲ ▲ (USP 1-May-2020)

Additional Release Testing of Clinical Site-Manipulated Cell Products

▲Cell-based ▲ (USP 1-May-2020) therapy products that undergo preparative steps or manipulations at clinical sites must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations determines whether additional release requirements or critical specifications must be added to those required immediately after initial manufacture.

Prerelease steps usually include the following:

- Physical inspection of the product, including product appearance (color, turbidity, particulates, or foreign matter), container integrity, temperature, and accuracy and convenience of labeling
- Review of process records and/or certificate of analysis
- For patient-specific products, verification of product labeling and records related to identity of the intended recipient

▲ ▲ (USP 1-May-2020)

Administration to Patients

▲ ▲ (USP 1-May-2020)

In all cases, adequate anesthesia and premedication must be carefully evaluated. For example, if the DMSO will remain in a thawed, cryopreserved cellular product, the patient may be given an antihistamine before administration. Pre-administration evaluation must also include assessment of concurrent therapies that may interact with the ▲cell-based advanced therapy or tissue-based ▲ (USP 1-May-2020) product to modify its effects. Some therapies may be adjunctive to the ▲cell-based advanced therapy

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or tissue-based,▲ (USP 1-May-2020) such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects.

DELIVERY OF CELL-BASED THERAPY TO PATIENTS

Some ▲cell-based advanced therapy or tissue-based▲ (USP 1-May-2020) products are patient specific because they are manufactured from a selected autologous or allogeneic tissue source, cells, or tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, and at least two people should verify the identity of the patient and patient-specific product immediately before administration.

▲▲ (USP 1-May-2020) A variety of delivery systems such as catheters, syringes, and intravenous (IV) lines are frequently used to administer cells to patients. Before clinical use manufacturers should ensure that these medical device components are compatible with the cells and formulation solutions. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

▲▲ (USP 1-May-2020)

Change to read:

STABILITY

General Considerations

▲Stability studies for cell-based therapies and tissue-based products should be based on a comprehensive understanding of the final therapeutic product and its intended use. Manufacturers should assess the stability of the final product, in-process hold steps, cell banks, critical raw materials, and reference standards.▲ (USP 1-May-2020)

Where feasible, stability testing should be carried out in accordance with the principles described in ICH guideline Q5C, presented in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩. Stability data should also be collected for bulk and other in-process materials that are stored before final processing and filling.

▲For some cell-based advanced therapies and tissue-based products, final lots may have small volumes and/or shelf lives. In such cases, stability protocols should be based on materials from multiple donors. Because it is difficult or unethical to obtain sufficient stability study cells or tissues from patients, cells or tissues from several sources such as normal donors, research tissue repositories, cadaveric sources, or well-characterized banked primary cells may be used in stability studies to validate storage, shipping, and expiration dating. These results must be interpreted with caution until data confirm that the actual product cells and "surrogate" source materials exhibit similar stability profiles.▲ (USP 1-May-2020)

For combination products that include cells and biomaterials, the stability of ▲all components when combined▲ (USP 1-May-2020) must be considered. When biodegradable scaffold materials are present, scaffold degradation should be considered in determining the stability and shelf life of the combination product.

Stability Protocol Development

Formal stability studies to support licensure and early-phase product stability information gathering should be detailed in a written plan that describes how stability data will be collected and analyzed to support the expiration date. ▲▲ (USP 1-May-2020)

Stability studies must verify that the storage conditions maintain the quality attributes of the product so that ▲it▲ (USP 1-May-2020) complies with stability specifications. These specifications may differ from release specifications, but they must address product potency. ▲▲ (USP 1-May-2020)

Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. ▲Such▲ (USP 1-May-2020) initial studies are useful for determining which assays are stability indicating, that is, the best indicators of product degradation. ▲▲ (USP 1-May-2020)

Shipping validations are a special type of stability study. ▲▲ (USP 1-May-2020) Typically, the product is packaged and shipped ▲(or subjected to simulated shipping)▲ (USP 1-May-2020) under normal and extreme conditions, and the material is tested before and after shipping to ensure that it still meets the product release requirements. As described in *Storage and Shipping* (below), special attention must be given to the specific thermal, mechanical, and radiological stresses ▲that cell-based therapies and tissue-based▲ (USP 1-May-2020) products will likely encounter.

Stability Challenge Conditions

▲Based on risk assessment, stability studies for cell-based advanced therapies and tissue-based products should include challenge conditions outside of the specified storage ranges, such as those encountered during periods of abnormal storage, shipping, or handling.▲ (USP 1-May-2020) Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation caused by shipping to hot or cold climates, hypobaric conditions in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite.

A short exposure to an environmental condition well outside of an established limit and a long exposure to one just outside of an established limit may be equally detrimental. ▲▲ (USP 1-May-2020) The effect of light on the stability-indicating profile should

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be investigated if it is scientifically warranted. Special attention should be given to products stored in fluids containing light-sensitive or light-reactive components that may give rise to cytotoxic by-products.

▲Accelerated aging studies are useful to characterize how the cell-based advanced therapy or tissue-based product degrades, and which assays are stability indicating.▲ (USP 1-May-2020) Such studies should be performed before formal stability studies begin so that the formal studies incorporate the validated stability-indicating assays into the protocol.

Change to read:

STORAGE AND SHIPPING

General Considerations

▲Storage conditions are chosen to preserve the quality of the cell-based advanced therapy or tissue-based product, so that product specifications are maintained throughout storage, shipping, and handling at the clinic. Before clinical trial use, initial studies must be conducted to determine acceptable storage, shipping, handling, and expiration dating.▲ (USP 1-May-2020) Once stability-indicating methods are developed and the final container–closure, storage, and shipping conditions are chosen, these conditions must be validated, as discussed in *Stability* (above).

▲▲ (USP 1-May-2020) The product ▲in its final container closure▲ (USP 1-May-2020) should be placed in a lightproof, leakproof ▲shipping▲ (USP 1-May-2020) container with adequate physical support to ensure stability and ▲prevent▲ (USP 1-May-2020) leakage during ▲▲ (USP 1-May-2020) shipment. Special consideration should be given to the ability of gas to permeate the shipping container, especially if the ▲cell-based therapy▲ (USP 1-May-2020) product is stored or shipped on dry ice or liquid nitrogen.

Storage

For each type of ▲cell-based▲ (USP 1-May-2020) therapy product, the manufacturer should establish product storage specifications and acceptable storage conditions, including temperature range or liquid nitrogen level. ▲Storage unit monitoring and▲ (USP 1-May-2020) alarm systems ▲should immediately notify manufacturing▲ (USP 1-May-2020) of unacceptable storage conditions. The stability of the product during routine storage should be monitored ▲and documented via▲ (USP 1-May-2020) a stability program (see *Stability*, above).

▲Cryopreservation is the main mode of long-term cell storage. Cell-based therapy products are most often cryopreserved using controlled-rate freezing, or equivalent procedures known to maintain viability. Freezer equipment should be validated and temperature mapped so that cells are stored at an appropriately low temperature. Product stability should be validated under the holding conditions at both the manufacturing facility and clinical site.▲ (USP 1-May-2020)

Many cell-based products cannot be cryopreserved. Because cells continue to metabolize during storage, their expiration period is short—on the order of hours or days▲—though the expiration date may be extended by increasing the volume of the storage medium, or by adjusting the storage temperature.▲ (USP 1-May-2020)

Shipping

▲Shipping containers and procedures for cell-based therapies and tissue-based products must ensure acceptable temperatures are maintained under conditions of actual use. These conditions include extremes of temperature inside and outside the shipping container, and other shipping challenges such as X-rays or mechanical vibration. Shipping studies should be conducted during product development in order to identify which stresses affect the product. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will protect the product against extreme temperatures and mechanical stresses and maintain the final container closure integrity. Shipping validations must ensure that the product meets quality specifications (including potency) once it reaches its final destination.▲ (USP 1-May-2020)

Most ▲cell and tissue-based▲ (USP 1-May-2020) products are shipped by commercial shippers or courier systems. ▲▲ (USP 1-May-2020) Commercial ▲aircraft shipments▲ (USP 1-May-2020) must obtain special permission in order to bypass scanning by airport X-ray equipment. Special attention should be paid to shipping container labels because both biohazard and patient-specific information may be required in specific areas of the packaging. Shipping validations must be conducted under predefined protocols with predetermined acceptance criteria to ensure that the product meets quality specifications (including potency) once it reaches its final destination.

Cryopreserved cell-based ▲therapy▲ (USP 1-May-2020) products are typically shipped to medical centers on dry ice or in liquid nitrogen dry shippers. Dry shippers may be preferable because temperature is more readily maintained and monitored. Dry shippers also allow continuous monitoring of the shipper's temperature, which can be collected and logged for up to 14 days. Dry ice and liquid nitrogen are both considered hazardous materials during shipping and must be labeled accordingly.

Change to read:

LABELING

Labeling of cell therapy products is regulated by the FDA under 21 CFR 201, 601, 610, and 1271. For biologics, 21 CFR 610 Subpart G outlines the requirements for container and package labeling. When possible, a full label should be affixed to the product container. ▲▲ (USP 1-May-2020) When partial labels are used, the container must be placed in a package that contains a label bearing all the items required for the package label. For containers that cannot accommodate any label, the container

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must be placed in a package that bears all the information required for a package label. ▲▲ (USP 1-May-2020) When affixed to the container the label should not impede inspection of the contents. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life.

▲Regulations in 21 CFR 610.62 refer to the position and prominence of the proper or United States Adopted Names (USAN) name in relation to a trade name.▲ (USP 1-May-2020)

Additional labeling requirements ▲in 21 CFR 1271.90▲ (USP 1-May-2020) apply because ▲some cell-based▲ (USP 1-May-2020) therapy products are also considered HCT/Ps. ▲▲ (USP 1-May-2020) For autologous cell therapies, the manufacturer is exempt from the requirements of determining donor eligibility. However, if the recommended testing for pathogenic or microbial contaminants is not performed before release, the label must contain the statement "FOR AUTOLOGOUS USE ONLY" or "NOT EVALUATED FOR INFECTIOUS SUBSTANCES." The label must also contain the Biohazard legend shown in 21 CFR 1271.3(h) with the statement "WARNING: Advise patient of communicable disease risks." For patient-specific products, the patient's full name, initials, or a combination of these must appear on the labeling to ensure that the product will be administered to the appropriate patient. ▲For materials containing human tissue, per AATB standards, transport package labels should also include prominent identification of contents as "DONATED HUMAN TISSUE" as well as prescribed storage conditions and transport expiration date (if applicable).

Regulations in 21 CFR 201.56 and 201.57 govern the content and format of the package insert.

Several groups have designed a standard for uniform labeling of cellular therapy products, ISBT 128, which defines the data structures and the placement of bar codes and their corresponding eye-readable text that appears beneath the bar code. In addition, ISBT 128 provides class names for different types of cellular products, and various other texts that must go on the labels. Although this voluntary ISBT 128 standard meets different organizations' requirements for labeling cellular products, it does not currently meet FDA regulatory requirements. Consequently, labels that comply with ISBT 128 must be supplemented with additional information required by FDA.▲ (USP 1-May-2020)

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CONSIDERATIONS FOR TECHNOLOGY TRANSFER

Transfer of the skills, knowledge, technologies, and methods of manufacturing necessary to create a cell or tissue-based product is essential to ensure that scientific and technological developments are accessible ▲and transferable to others.▲ (USP 1-May-2020) Some general considerations for technology transfer activities are summarized below.

The process of developing a ▲cell-based therapy or tissue-based product▲ (USP 1-May-2020) is complex and often involves several rounds of technology transfer throughout the product's life cycle. Some examples of technology transfer activities include: from bench research to translational research; transfer from research and development to GMP-compliant manufacturing; and change in manufacturing facility (for example, from in-house manufacturing to a contract manufacturer).

Manufacturers should anticipate the need for technology transfer during the research and development stage of a cell or tissue-therapy process. This should result in good documentation practices for product research and development, including testing procedures. Data and results should be retained in the format of development reports or technical reports to provide historical information that can be referenced and used in regulatory filings. Critical raw materials, procedures, and equipment should also be identified during technology transfer. Product and process development progress should be monitored against milestones established as part of risk assessment and gap identification in the technology transfer plan. *Table 4* provides an overview of the steps involved in technology transfer.

Table 4. Technology Transfer—Fundamental Steps

Preparation	• Define the scope, strategy, and risks associated with the project that will be transferred • Identify overall gaps and process transferability • Assess availability of documentation such as manufacturing and testing procedures, sampling plans, in-process and final product data and specifications, material specifications (including source, testing requirements, and quantities required for a manufacturing procedure or test procedure), equipment specifications, specialized training requirements, facility requirements, and infrastructure requirements • Establish a governance body consisting of leads, experts, and mentors from both the sending and receiving sides; determine responsibilities for each group and individual • Define communication and reporting channels • Identify performance measurements, milestones, and timelines
Development and Implementation	• Establish a risk management plan • Establish a technology transfer master plan • Develop a training plan • Establish documents at the receiving site (specifications, SOPs, batch records, and standard test methods) • Train operations, quality, and support personnel for sustainability • Qualify materials and vendors • Establish and execute equipment comparability/suitability protocols • Calibrate equipment at receiving site • Qualify personnel, equipment, and facility at receiving site (includes execution of aseptic process validations, sterile media fills, and cleaning validations) • Establish and execute methods/assay qualifications • Establish a product stability program • Perform engineering and consistency/qualification runs • Assess need to establish comparability and prepare regulatory filings • Establish and execute shipping qualifications

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Table 4. Technology Transfer—Fundamental Steps *(continued)*

Maintenance	• Collect and trend process/product data • Monitor product stability • Manage change control • Train and requalify personnel • Recalibrate and requalify equipment • Update regulatory filings

The ultimate goal of technology transfer is for the recipient to consistently reproduce a process ▲in order▲ (USP 1-May-2020) to make a comparable product in compliance with regulations. It is not atypical for manufacturers to develop and implement process improvements during early stages of technology transfer to support scale-up and manufacturing for Phase I/II clinical trials. However, ▲for▲ (USP 1-May-2020) technology transfer ▲during▲ (USP 1-May-2020) Phase III studies, pivotal trials, or commercial manufacturing, changes to the process or product should be avoided because they could require additional clinical studies and adversely affect time to market.

Change to read:

REGULATIONS AND STANDARDS

The Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Public Health Service Act (PHS Act) provide the legal framework for FDA regulation of biological products, including cell-based therapy products. A list of frequently used terms in regulation of cellular-therapy products is presented in *Table 5*. In 1993 FDA provided notice that it intended to regulate cellular and gene-therapy products as biological products (*Federal Register* 1993;58:53248–53251). The FDA defined somatic ▲cellular-▲ (USP 1-May-2020) therapy products as autologous (i.e., self), allogeneic (i.e., intraspecies), or xenogeneic (i.e., interspecies) cells that have been propagated, expanded, selected, pharmacologically treated, or otherwise altered ex vivo for administration to humans for the prevention, treatment, cure, diagnosis, or mitigation of disease or injuries. For other biological products and drugs, clinical trials involving somatic cellular therapy products must be initiated under an investigational new drug (IND) application. After a sponsor submits sufficient evidence of product safety and clinical effectiveness, FDA approval can be obtained for marketing in the form of a BLA or PMA.

As defined by the FDA, cellular therapy products are considered to be drugs, biological products but also HCT/Ps that are regulated under Section 351 and/or Section 361 of the PHS Act. This means that cell-based therapies are subject to cGMP (21 CFR 210 and 211), Biologics Product regulations (21 CFR 610), and HCT/P regulations (21 CFR 1271) including cGTP.

In recent years, the FDA has issued a number of regulations and guidance documents for human cell and tissue products (see *Appendix* and www.fda.gov/cber/). Of particular importance are the regulations at 21 CFR 1271 that establish a tiered, risk-based approach for HCT/Ps. In this regulatory framework, many conventional human cells or tissues are not subject to premarket approval and have only to comply with GTPs, including donor eligibility. This lower tier of regulatory oversight is intended to prevent the introduction, transmission, or spread of communicable disease. When human cells or tissue are the starting material for the creation of a novel cell-based product, additional regulatory requirements are applicable. This higher tier of regulatory oversight includes compliance with GMPs, biological product standards, and premarket approval (see 21 CFR 1271.10). In almost all cases, the cell-based products described in this chapter should comply with the higher tier of regulatory oversight.

In addition to cellular therapy-specific regulations and guidance, many general guidelines such as those related to aseptic processing, GMP expectations during development, process validation, and others are relevant and applicable (see www.fda.gov). Additionally, ICH has issued guidance documents for qualifying ▲cell-based therapy▲ (USP 1-May-2020) and tissue-based products (see *Appendix* and www.ich.org). Some of the guidelines and concepts in these documents are reproduced in *USP–NF*.

The regulatory pathway for ▲cell-based therapy▲ (USP 1-May-2020) products parallels that of pharmaceuticals, and as the product moves from early research through pivotal trials and finally marketing approval, the degree of manufacturing control becomes increasingly stringent. This has implications for the manufacturing unit and may dictate that the site be moved. Standards-setting organizations encourage the use of a fully functional quality unit to oversee manufacturing progress. Information is available on the FDA website, along with references to groups charged with guiding the medical community and the manufacturing unit during development.

In addition to *USP* general chapters and monographs for ▲cell-based therapies and tissue-based products,▲ (USP 1-May-2020) a number of professional standards-setting organizations (see *Table 6* and *Appendix*) have worked closely with regulatory authorities to develop standards and practices. These organizations ensure that standards are current and comply with governmental regulations. Such standards are a supplemental source of knowledge in identification of donors, donor screening and testing, product collections, processing of cellular products, administration, adverse event reporting, and follow-up after treatment. The AATB has developed guidelines for sourcing allogeneic tissue. Over the years various organizations have tried to harmonize standards, including the development of common information circulars that can be compared with package inserts. At present, however, compliance with one organization's standards does not ensure compliance with those of any other organization.

Many benefits accrue to manufacturing facilities that participate in voluntary standards programs. Professional standards-setting organizations participate in educational workshops and disseminate information about operational issues. They also maintain close surveillance of FDA activity and training of inspectors. Further, the FDA relies on accreditation by voluntary standards program, and the FDA's unannounced inspections have led to an increasingly high level of compliance in laboratory and clinical settings and has also undoubtedly increased patient safety. Third-party payors and hospital-ranking services have begun to use accreditation reports in their evaluation of quality programs.

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Table 5. Frequently Used Terms in Regulation of Cellular-Therapy Products

Term	Definition
351 products	Regulated under Section 351 of the PHS Act
361 products	Regulated under 21 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products
BLA	Biologics ▲License▲ (USP 1-May-2020) Application
▲CAR-T	Chimeric Antigen Receptor T-cells▲ (USP 1-May-2020)
CBER	Center for Biologics Evaluation and Research
CDRH	Center for Devices and Radiologic Health
▲c▲ (USP 1-May-2020)GMPs	▲current▲ (USP 1-May-2020) Good manufacturing practices
GTP	Good tissue practices, 221 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products
▲HCT/Ps	Human cells, tissues, and cellular and tissue-based products▲ (USP 1-May-2020)
IDE	Investigational Device Exemption. An IDE allows the investigational device to be used in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PMA) application or a Premarket Notification [510(k)] submission to the FDA.
IND	Investigational New Drug. An IND is a request for FDA authorization to administer an investigational drug to humans. IND regulations are contained in 21 CFR 312.
▲MCB	Master cell bank▲ (USP 1-May-2020)
PMA	Premarket approval
▲WCB	Working cell bank▲ (USP 1-May-2020)

Table 6. Cellular Therapy Product Standards-Setting Organizations

AABB	AABB, formerly known as the American Association of Blood Banks, is an international association representing individuals and institutions involved in activities related to transfusion and cellular therapies, including transplantation medicine.	www.aabb.org/
AATB	The American Association of Tissue Banks is an educational and scientific, tax-exempt organization that facilitates the provision of transplantable tissues of uniformly high quality to meet national needs. AATB publishes standards to ensure that the conduct of tissue banking meets acceptable norms of technical and ethical performance. AATB conducts an accreditation program for establishments that retrieve, process, store, or distribute human tissue for transplant. A certification program is administered for tissue-bank personnel to ensure that tissue-banking activities are performed in a professional manner consistent with the standards of the association.	www.aatb.org/
ASTM	ASTM International (ASTM), originally known as the American Society for Testing and Materials, is one of the largest voluntary standards-development organizations in the world and provides technical standards for materials, products, systems, and services. ASTM International standards are used in the information infrastructure that guides design, manufacturing, and trade in the global economy.	www.astm.org/
FACT	The Foundation for the Accreditation of Cellular Therapy is a nonprofit corporation co-founded by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT) for voluntary inspection and accreditation in the field of cellular therapy.	www.factwebsite.org/
NMDP	The National Marrow Donor Program is a nonprofit organization that operates the federally funded registry of volunteer hematopoietic cell donors and umbilical cord blood units in the United States.	www.nmdp.org/
ICCBBA	The International Council for Commonality in Blood Banking Automation was established and given the responsibility for implementation and management of the ISBT 128 standard, a system for identification, labeling, and processing of human blood, tissue, and cellular-therapy products using an internationally standardized system.	www.iccbba.org/

Change to read:

GLOSSARY

Adventitious agent: A foreign material that is introduced inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

Allogeneic: From an unrelated member of the same species but with a different genotype.

Ancillary materials: Components used during manufacturing that should not be present in the final product. Examples: growth factors, cytokines, monoclonal antibodies, cell-separation devices, and media components.

Apheresis: Procedure of withdrawing blood from a donor, removing select components (e.g., platelets or leukocytes), and transfusing the remainder into the donor.

Autologous: From one's own body.

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Bioassay: Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*.)

Biological product: Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries in humans. (The term "analogous product" has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

Biotechnology: Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

B Lymphocytes (B cells)**:** A class of lymphocytes that produce antibodies and are derived from bone marrow.

Bone marrow cells: A variety of undifferentiated cells (stem cells) and differentiated cells (lymphocytes, granulocytes, erythrocytes, and platelets) found in the internal cavities of bones or bone marrow.

Bone marrow transplantation: Transplantation of bone marrow cells that are capable of maintaining the hematological functions indefinitely. Technique used in the treatment of immunological disorders (severe combined immune deficiencies such as ADA deficiency), hematological disorders (anemia), metabolic disorders (Gaucher disease), and malignant diseases (leukemia, lymphoma, or solid tumor).

CD34: Cluster of differentiation cell-surface marker 34. CD34 is a protein that distinguishes stem and progenitor cells from more mature blood cells.

Cell lines: Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or be immortalized (made to replicate indefinitely).

Cellular therapy: Therapy that uses whole cells to treat a disease, condition, or injury.

cGMP: Current good manufacturing practice.

Chondrocytes: Cells that produce the components of cartilage.

Clonal: Genes, cells, or entire organisms derived from and genetically identical to a single common ancestor gene, cell, or organism.

Clonogenic assay: Procedure based on the ability to give rise to a clone of cells.

Combination products: Therapeutic products that combine drugs, devices, and/or biological products.

Culture medium: The liquid that covers cells in a culture vessel and contains ingredients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.

Cytokine: Any factor that acts on cells; usually a protein that promotes growth.

Cytoplasm: Cellular material that is within the cell membrane and surrounds the nucleus.

Cytotoxic: Able to cause cell death.

Dendritic cells: Cells that sensitize T cells to antigens.

Differentiation: A process of biochemical and structural changes by which cells become specialized in form and function.

Enzyme-linked immunosorbent assay (ELISA)**:** An immunoassay that uses an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

Embryonic stem cell, human (hESC)**:** Stem cell derived from the inner cell mass of the blastocyst.

Endothelial cells: Epithelial cells of mesodermal origin that line the internal cavities of the body, such as heart and blood and lymph vessels.

Engraftment: Process whereby cells, tissues, or organs are implanted or transplanted into another organism. Refers to both the mechanical and the biological processes necessary to have a fully functional graft.

Epidermal: Pertaining to the outermost and nonvascular layer of the skin derived from embryonic ectoderm.

Epithelial cells: Cells from the linings of various organs, e.g., respiratory, intestinal, or vascular epithelial cells.

Ex vivo: Outside of the living body. Refers to a medical procedure in which an organ, cells, or tissue are taken from a living body for a treatment or procedure, and then returned to the living body.

Feeder cells: Cells used in co-culture to maintain pluripotent stem cells. For hESC, typical feeder layers include mouse embryonic fibroblasts or human embryonic fibroblasts that have been treated to prevent them from dividing.

Fibroblasts: Connective tissue cells that have the capacity to produce collagen.

Fluorescence-activated cell sorter (FACS)**:** A machine that sorts cells based on fluorescent markers attached to them.

Formulated: Prepared in accordance with a prescribed method or conditions.

Graft-versus-host disease: Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

Granulocyte: One of three types of white blood cells. These cells digest bacteria and parasites.

Granulocyte–macrophage colony-stimulating factor (GM CSF)**:** A natural hormone that stimulates white blood cell production, particularly that of granulocytes and monocytes.

Growth factors: Factors responsible for regulatory cell proliferation, function, and differentiation.

Hemacytometer: A device used to manually count cells.

Hematopoietic: Pertaining to or affecting the formation of blood cells.

Hematopoietic stem cells: Stem cells that give rise to all red and white blood cells and platelets.

Hepatocytes: The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells are generally not dividing, but when injured they can divide and regenerate until the injured cells are replaced.

Human leukocyte antigen (HLA)**:** Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

Immunoassay: Technique for identifying substances based on the use of antibodies.

Immunofluorescence: Technique that combines an antibody detection strategy with a fluorescent label for visualization often used in combination with microscopy or fluorescence activated cell sorting.

Immunogenic: Substance capable of inducing an immune response; a form of antigen that induces an immune response, as opposed to a tolerogen that induces tolerance.

In vitro: In the laboratory (outside the body). The opposite of in vivo (in the body).

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In vivo: Procedure performed in the living organism.
Islet cells: β-islet cells of the pancreas that secrete insulin.
Keratinocytes: Differentiated epidermal cells that constitute the top layer of cells in the skin.
Lineage (committed progenitor cells, differentiated cells)**:** Specific path of cell differentiation that can be traced to a single cell of origin.
Macrophage: Any of many forms of mononuclear phagocytes that are found in tissues and arise from hematopoietic stem cells in the bone marrow.
Mesenchymal stem cells: Multipotent stem cells that can differentiate into a variety of cell types.
Monoclonal antibodies: Antibodies that are derived from a single cell clone.
Myocytes: Fundamental cell units in the muscle. Target cells for insertion of genes that encode secretory proteins.
Natural killer cells (or NK cells)**:** Cytotoxic lymphocytes that constitute a major component of the innate immune system.
Neuronal stem cells: Stem cells found in neural tissue that can give rise to neurons and glial cells.
Osteogenic cells: Derived from or involved in the growth or repair of bone.
Passage: The process in which cells are disassociated, washed, and seeded into new cultures after a round of cell growth and proliferation. The number of passages is a good indication of the age of the cultures and expected stability.
Process validation: Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.
Polymerase chain reaction (PCR)**:** Technique to amplify a target DNA or RNA sequence of nucleotides by cycles of polymerase-based copying, resulting in geometric increases in copy number.
Potency: A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.
Progenitor cell: Parent or ancestral cell, usually one that is already committed to differentiate into a specific type or lineage of cells.
Regenerative medicine: An emerging interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function using a combination of approaches including, but not limited to, the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering, and the reprogramming of cell and tissue types.
Serum-free: Refers to cell growth medium that lacks a serum component.
▲**SIS:** Subintestinal submucosa.▲ (USP 1-May-2020)
Somatic cells: Cells other than the germ cells.
Stem cell: Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.
Supravital dye: A dye that stains only living cells.
Suspension culture: Growth, in suspension, of cells not requiring attachment to substrate in order to undergo cell division.
T cells: Lymphocytes that acquire functional repertoires and the concept of self in the thymus and are responsible for cell-mediated immunity. There are several subsets of T cells (helper T cells, suppressor T cells, and cytotoxic T cells).
Umbilical cord blood stem cells: Stem cells derived from the blood that remains in the placenta and in the attached umbilical cord after childbirth.
Undifferentiated cells: Cells that have not yet developed into a specialized cell type or tissue.
▲**Working standard:** In-house or secondary standard that is qualified against and used in place of a reference standard.▲ (USP 1-May-2020)
Xenogeneic: From a different species.
Xenotransplantation: Transplantation of organs from one species to another (e.g., from pigs to humans).

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APPENDIX

Lists of Relevant Regulatory References

Cellular therapies and cell-therapy components are regulated by FDA as biological products. The general requirements are listed in national laws and international guidance. In the US, national requirements are codified in different sections of 21 CFR, and additional recommendations are available in FDA guidance documents. International guidance documents are available from the ICH, the European Agency of Medicines (EMA), and the World Health Organization (WHO). Although guidance documents from the ICH are well referenced in this chapter, those from WHO and European Medicines Evaluation Agency (EMEA) are not, and manufacturers of cellular or tissue-based products intended for markets outside the United States are advised to refer to relevant guidances from relevant nations. Beyond *USP* chapters referenced in this chapter, the following lists include regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance.

Code of Federal Regulations (CFR)

▲Regulations	Scope
21 CFR 201	Labeling
21 CFR 210	cGMP for drugs
21 CFR 211	cGMPs for final products
21 CFR 600.3	Definitions of important terms for biological products

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▲Regulations	Scope
21 CFR 601	Requirements for marketing authorization of biologics under BLA
21 CFR 610	Identity, purity, potency, and microbial safety testing requirements for biological products
21 CFR 610.12	Sterility
21 CFR 610.60–68	Product and container labeling requirements for biological products
21 CFR 820	Quality systems for products regulated as medical devices
21 CFR 1271	Good tissue practices (GTPs)
45 CFR Part 46	Department of Health and Human Services, Part 46, Protection of Human Subjects▲ (USP 1-May-2020)

FDA Guidance Documents

- ▲▲ (USP 1-May-2020) *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*, ▲April 2008. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/xenotransplantation/ucm092705.pdf.▲ (USP 1-May-2020)
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National and International Regulatory Documents

- The United States Consensus Standard for the Uniform Labeling of Cellular Therapy Products using ISBT 128. Available at: http://www.iccbba.org/.

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⟨1047⟩ GENE THERAPY PRODUCTS

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INTRODUCTION

Gene therapy products allow administration of nucleic acids to modify the genetic material of cells. Gene therapy products can be broadly classified based on the approach to delivery and include the following: 1) viral vectors [viruses that harbor the gene(s) of interest but usually without the mechanism to self-replicate in vivo]; 2) nucleic acids in a simple formulation (naked DNA); and 3) nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Where introduction of nucleic acid to cells takes place ex vivo, the cell population that is administered becomes the gene therapy product. Guidance specific to the manufacturing, processing, characterization, and administration of cell-based products is provided in ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩.▲ (CN 1-May-2020)

Decisions regarding the choice of a gene vector can be complex (see *Design Considerations for Gene Vectors*). The viruses most commonly used include murine retroviruses, human adenoviruses, and human adeno-associated viruses (AAVs). It is inherent in the definition of gene therapy in this chapter that the administration of nucleic acid through transduction is expressed as RNA and then as protein. Examples of gene therapy products are shown in *Table 1*.

Table 1. Examples of Gene Therapy Products

Categories or Strategies	Indication: Administered Product
Gene replacement Short-term Long-term	Cardiovascular disease: growth factor vector on a biocompatible scaffold[a] Cystic fibrosis: transmembrane conductance regulatory vector Hemophilia: factor VIII or IX vector
Direct cell killing	Cancer: recombinant oncolytic viruses
Immunotherapy	Cancer: autologous tumor cells transduced with cytokine or other immunomodulatory genes; lymphocytes transduced with receptors for tumor antigens Arthritis: gene-modified autologous lymphocytes
Conditionally lethal genes[b]	Cancer (solid tumor): thymidine kinase (TK) or cytosine deaminase (CD) vector into tumor cells Graft-versus-host disease (GVHD): TK or CD vector transduced into donor T cells
Gene disruption via antisense RNAs, ribozymes, and inhibitory RNAs expressed via a vector	Cancer: anti-oncogene vector Cytomegalovirus retinitis: anti-viral vector Human immunodeficiency virus (HIV): autologous lymphocytes transduced with antiviral ribozyme vector
Intrabodies	Cancer or HIV: vector encoding single-chain antibody to a tumor protein or a viral protein, respectively

[a] This product promotes formation of new blood vessels.
[b] Cells with conditionally lethal genes as well as their neighboring cells are killed after the administration of a second drug in vivo. For TK, the drug is gancyclovir. For CD, the drug is 5-fluorocytosine.

CHAPTER PURPOSE AND ORGANIZATION

Clinical uses for gene therapy products, their manufacturing processes, and analytical schemes for determining identity, dose, potency, purity, and safety are rapidly evolving, and are as diverse as the products themselves. This chapter summarizes the issues and best current practices in the manufacturing, testing, and administration of gene therapy products. Usually *USP* chapters focus on materials that are commercially available. This chapter, however, not only discusses products for commercial applications, but it also addresses the production of clinical trial materials. When different approaches are options for clinical trial material compared to those used for commercial product, this is discussed.

Where appropriate, reference is made to applicable guidance including International Council for Harmonisation (ICH) quality guidelines because the principles apply even though gene therapy products may be outside the official scope. A list of regulatory

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and guidance documents applicable to gene therapy is presented in the *Appendix*, together with a list of terms commonly used in the gene therapy field. The traditional compendial perspective is to develop public standards that can be applied to a particular final product without providing production details. This chapter attempts to specify when traditional methodologies or standards can be adapted.

This chapter is extensive because of the diverse nature of the products and the special considerations that they require. Manufacturing has been divided into two sections: the first discusses general aspects of manufacturing and process development, and the second discusses vector design and class-specific topics. *On-Site Preparation and Administration* follows the manufacturing sections because the handling of these products at the clinic often requires facilities and expertise not found in a typical hospital. Other manufacturing-related sections include: *Analytical Methods*; *Stability*; *Storage and Shipping*; and *Labeling*. *Regulations and Standards* summarizes existing guidelines and highlights the need for the development and validation of new methodologies to assess product quality. The *Glossary* lists and defines the terms and abbreviations used in this chapter and those commonly employed in this field.

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MANUFACTURING OVERVIEW

Introduction

The manufacturing of gene therapy products has been divided into two sections. This section discusses five topics that apply to manufacturing of all gene therapy products: 1) raw materials, 2) characterization of banked materials, 3) in-process controls, 4) specifications, and 5) validation considerations. The second section, *Manufacturing of Gene Therapy Products*, addresses manufacturing of gene therapy vectors, both viral and nonviral, and discusses the design of gene vectors in detail.

All the general principles of current good manufacturing practice (cGMP) outlined by the FDA in 21 Code of Federal Regulations (CFR) 210, 211, 600s (especially 21 CFR 610), and 820, as well as other *USP* chapters apply to the manufacturing of gene therapy products. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of cGMP. cGMPs are applied throughout clinical development. Typically, the extent of control increases as clinical development progresses, and full cGMP compliance is expected by initiation of manufacturing in support of Phase III clinical trial(s). The facility and equipment should be carefully designed, built, and validated to support the manufacturing process and to maintain the required product/facility segregation. Preventive maintenance and calibration should be performed routinely on critical equipment. Incubators, bioreactors, and freezers should be fitted with alarm systems that can remotely signal failure. Quality systems should be established to ensure manufacturing is consistent and in control. Systems include but are not limited to the following: change control, document control, environmental monitoring, training, validation master plans, raw material testing and release, vendor approval, product testing and release, stability testing, and corrective/preventive action (CAPA).

Ancillary Materials

A wide variety of raw materials, including ancillary materials, may be used in manufacturing. Raw materials may include complex substances such as cells, tissues, biological fluids, growth factors, and monoclonal antibodies. Some of these materials may remain in the final therapeutic product as active substances, cryoprotectants, or excipients. An ancillary material exerts an effect on a therapeutic material (for example, a cytokine may activate a population of cells) but is not intended to be present in the final therapeutic product. The quality of raw materials used in the production of a gene therapy product can affect the safety, potency, and purity of the product. Therefore, qualification of this type of materials is necessary to ensure the consistency and quality of all gene therapy products. Activities involved with raw material qualification will change as products move through various stages of clinical development and on to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. A qualification program for raw materials used in the manufacturing of gene therapy products should address each of the following areas: 1) identification and selection, 2) suitability for use, 3) characterization, 4) animal-derived components, and 5) quality assurance. For all raw materials, it must be considered when and where each is used in the manufacturing process because this can help define selection criteria. *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩ should be consulted for specific information about implementing an appropriate qualification program for these materials. Other *USP* chapters provide information about the qualification and standards of specific ancillary materials (e.g., *Bovine Serum* ⟨1024⟩, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩, and *Growth Factors and Cytokines Used in Cell Therapy Manufacturing* ⟨92⟩).

Characterization of Cell and Virus Banks

CELL BANKS

A cell bank is a collection of vials containing cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single cell clone. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although more tiers are possible. The MCB is manufactured in accordance with cGMP and preferably is obtained from a qualified repository source (source free from adventitious agents) with known and documented history. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to consistency of production of clinical or licensed product batches because the starting cell material is always the same. Cell banks used for the preparation of virus banks or clinical product should be suitably characterized before use. Aspects of cell banking and validation are addressed in

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⟨1046⟩, *Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of rDNA-Derived Protein Products* ⟨1048⟩, and *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

VIRUS BANKS

The master virus bank (MVB) is similar in concept to the MCB because it is derived from a single production run and is uniform in composition. The working virus bank (WVB) is derived directly from the MVB. As with the cell banks, the purpose of a virus bank is to have a consistent source of virus that is shown to be free of adventitious agents for use in production of clinical or product batches. In keeping with cGMP regulations, testing of the cell bank that will be used for production of the virus banks, including quality ▲control▲ (USP 1-May-2020) testing, should be completed before the use of this cell bank for production of virus banks.

QUALIFICATION

Cell and viral bank characterization is an important step toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. Testing to qualify the MCB or MVB is performed once, and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB or MVB should be established. It is important to document the MCB and MVB history, the methods and reagents used to produce the bank, and the storage conditions. All the raw materials required for production of the banks—media, sera, trypsin, and similar substances—must also be tested for adventitious agents.

QUALIFYING THE MASTER CELL BANK

The FDA *Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy* (March 1998) provides specific recommendations for qualifying MCBs. Additional guidance is provided in ICH Q5D. A description and history of the cell line is required, along with a description of the freezing process, storage conditions, and the number of vials prepared. The identity of the cells should be analyzed by genotypic and/or phenotypic markers. For MCB containing vector sequences, the presence and integrity of the vector should be demonstrated using molecular assays (restriction endonuclease mapping and/or nucleic acid sequencing) and/or measurement of vector gene expression. Purity must be analyzed to exclude bacterial, mycoplasma, fungal, and viral contamination (other than vector sequences). Freedom from adventitious viruses should be demonstrated using both in vitro and in vivo virus tests and appropriate species-specific tests such as the mouse antibody production (MAP) test. Special attention should be given to the detection of replication-competent virus (RCV) arising from recombination of the vector and viral sequences. The MCB is further qualified by tests conducted on cells (from the MCB or WCB) expanded to the limit of in vitro cell age for production.

QUALIFYING THE MASTER VIRUS BANK

Testing of the MVB is similar to that of the MCB and should include testing for freedom from adventitious agents in general (such as bacteria, fungi, mycoplasma, or viruses) and for organisms specific to the production cell line, including RCV. Identity testing of the MVB should establish the properties of the virus and the stability of these properties during manufacture.

QUALIFYING THE WORKING CELL OR VIRUS BANK

Characterization of the WCB or WVB is generally less extensive and requires the following: 1) testing for freedom from adventitious agents that may have been introduced during generation of the WCB, 2) testing for RCV, if relevant, 3) routine identity tests to check for cell line cross-contamination, and 4) demonstration that aliquots can consistently be used for final product production. This assumes that the WCB and WVB were prepared in a controlled environment using media and equipment that were screened appropriately for adventitious agents. If not, additional release testing is required.

In-Process Controls

Manufacturing processes should have well-defined go–no go decision criteria that are applied to key in-process intermediates and are used to pool material that has been processed through a step in several sublots. Quality must be built into the product as well as tested during batch release. In-process controls are the assays or tests that are performed to ensure that the in-process intermediate is of sufficient quality and quantity to ensure manufacture of a quality final product. Examples of in-process controls are listed in *Table 2*. The main reason for performing the in-process control is to ensure that the correct product with anticipated quality and yield is obtained. Intermediate in-process material that fails to satisfy the in-process control criteria should not be used for further manufacturing. This material may be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications before it can undergo further manufacturing. If several sublots will be pooled for further processing, sublots that fail to satisfy the criteria should not be included in the pool, even if the pool containing these failed sublots would pass the in-process assay criteria. During clinical development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. The information from these runs is also used to set the criteria for the selected assays. In-process controls are performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended, and actions should be taken to correct problems as they arise.

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Table 2. Examples of In-Process Control Applications

Type of Product	Attribute to Control
Viral gene therapy	Quantity of virus after virus culture Specific activity of virus in fractions after column chromatography Quantity of host-cell DNA in fractions after column chromatography
Nonviral gene therapy	Optical density or change in oxygen consumption during culture Amount and form of plasmid before culture harvesting Amount and form of plasmid after extraction steps Amount of pyrogen or endotoxin after extraction steps in plasmid pool

Specifications

The specification for a gene therapy product should be chosen to ensure the safety and efficacy of the product before use. Selected tests should be product-specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and gene products should follow the principles outlined in ICH Q6B and should reflect the FDA's *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*.

Establishing specifications for a drug substance and drug product is part of an overall manufacturing control strategy that includes control of raw materials, excipients, and cell and virus banks; in-process testing; process evaluation and validation; stability testing; and testing for consistency of lots. When combined, these elements provide assurance that the process is in control and that the key quality attributes of the product are maintained. Appropriate specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Specifications for each product and its ingredients should be developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to safety and efficacy assessments. Specifications should accommodate the inherent variabilities exhibited by the production process and by the assay. Some lot-release specifications typically applied to biologics may require re-examination for these product types.

The procedures in a specification for the product are anchored by appropriate reference standards. The reference standard for the product ensures that the product, as measured by the release assays, does not change significantly over time. The reference standard is manufactured using the same process as used for clinical production and is subject to all in-process and final release testing. In addition, the reference standard may be subjected to additional characterization not typically performed as part of lot release. The reference standard need not be stored at the same dose, formulation, or temperature as the product, but the stability of this reference standard should be determined. The reference standard verifies that a test produces acceptable results (passes its system suitability tests). A specific assay standard (working standard) can be used in the test, but it should be calibrated against and behave like the reference standard. Changing to a new reference standard (lot) should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted.

Additional specifications may be needed to produce a safe and effective gene therapy product. These might relate to some of the controls and action limits used to maintain standards and consistency for raw materials, excipients, and the manufacturing process (see *Ancillary Materials* and *In-Process Controls*). Specifications should be established to allow acceptance of raw materials and excipients used in the final formulation of the product. In addition, tests should be performed at critical decision steps during manufacture or at points where data serve to confirm consistency of the process. In-process release specifications should be established for each control step. Heterogeneity can result from the manufacturing process or storage of the product. Therefore, the manufacturer should define the pattern of heterogeneity within the product and should establish limits that will maintain the therapeutic efficacy and safety of the product.

In some cases, specifications may be established for lot release as well as for shelf life. As discussed in ICH Q5C and *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩, the use of different specifications should be supported by sufficient data to demonstrate that the clinical performance is not affected. Acceptance criteria should be established and justified on the basis of data obtained from lots used in preclinical and clinical studies and lots used for demonstration of manufacturing consistency and on the basis of relevant development data such as those arising from validated analytical procedures and stability studies. Acceptance criteria should also be correlated with safety and efficacy assessments.

Once specifications have been established, test results should be trended. Results that are out-of-specification (OOS)—or even those that are out of trend—should be investigated before the material is considered for further processing. The purpose of an investigation is to determine the cause of the discordant result. The FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that the product is not within the specification, the lot should be rejected. In unique situations, a product that does not meet all specifications may have to be administered to a patient. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results.

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Considerations for Validation

The potential for wide biological variation in gene therapy products, particularly for patient-specific treatments, affects the validation effort. Nevertheless, the basic principles of process validation for any biological product, including recommendations by ICH, FDA guidance documents, *Validation of Compendial Procedures* ⟨1225⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩, apply to the validation of most gene therapy products. Guidelines for validating viral vaccines can be relevant to gene therapy processes that produce viral vectors. The hold steps in a manufacturing process should be validated to ensure that in-process intermediates are within specification and that the quality attributes of the final product are maintained. Product-release assays should be validated before production of the materials for Phase III pivotal clinical trials.

Process validation demonstrates that the unit operations of the manufacturing process perform consistently and can generate a quality product that meets specifications. Because biological processes are prone to variability, the consistency and robustness of the manufacturing process should be determined by validating the process on at least three lots. Process validation issues pertinent to cell-based products are addressed in ⟨1046⟩.

If possible, the process should be validated for virus clearance according to principles discussed in ICH Q5A. If this is difficult because of the nature of the gene therapy vector (e.g., enveloped virus), additional characterization of cells and animal-derived components used in the production process should be considered. If the gene therapy product is manufactured in a multiproduct facility, validate cleaning of multiproduct equipment and rooms to demonstrate the effectiveness of cleaning agents to inactivate or remove virus.

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MANUFACTURING OF GENE THERAPY PRODUCTS

Introduction

Principles for the production of pharmaceutical or biological products are also relevant to the production of gene therapy vectors for use in humans. The same cGMP requirements are applied to ensure that a high-quality product is delivered to the patient. Because of the nature of gene therapy manufacturing systems, most manufacturers face development issues such as scalability, yield, cost efficiency, and product stability.

Most gene therapy vectors have been produced only in relatively small batches necessary to meet the needs of early clinical trials conducted in small numbers of patients. However, the promise of gene therapy in larger patient populations has led to progress in large-scale production and purification technology. This section focuses on designing vectors for gene therapy and choosing a suitable production technology.

Design Considerations for Gene Vectors

TYPES OF VECTORS

A typical gene therapy vector is composed of the following: 1) the vector backbone; 2) a promoter; 3) the therapeutic gene, either as cDNA or genomic sequence; and 4) a polyadenylation signal. A wide array of viruses—including murine and human retroviruses, adenoviruses, parvoviruses such as AAV, herpes viruses, poxviruses, toga viruses, and nonviral plasmid therapy systems—have been developed for gene therapy applications. These vectors (see *Table 3*) differ greatly in terms of their capacity to deliver genetic material and the duration of expression. Some viral vectors preferentially target dividing cells, but others are capable of transducing both dividing and nondividing cells. There are significant variations in transgene capacity (i.e., there are limitations on the size of the foreign DNA fragment that can be incorporated into the vector genome). The level, timing, and duration of gene expression required for a gene therapy product depends on the clinical indication. Low-level, long-term gene expression may be required for some diseases, including adenosine deaminase (ADA) deficiency or type A and type B hemophilia. High-level, short-term expression may be more appropriate for cancer when genes that induce apoptosis are used or for cardiovascular disease when preventing hyperproliferation of smooth-muscle cells that may impede restenosis of saphenous vein grafts.

Table 3. Types of Gene Vectors

	Viral							Nonviral
Family	Retroviridae		Adenoviridae	Parvoviridae	Herpesviridae	Togaviridae	Poxviridae	—
Examples species	Murine Leukemia Virus	HIV	Adenovirus	AAV	Herpes Simplex Virus	Sindbis	Poxvirus (Vaccinia)	Plasmid derived
Vector Characteristics								
Insert size limit	8 kb	8 kb	4.3–34 kb	4–5 kb	40–150 kb	5 kb	25–50 kb	12 kb
Chromosome integration	Yes	Yes	No, episomal	Can be integrated or episomal	Can be integrated or episomal	No	No	Yes, but at very low frequency
Therapeutic expression	Stable	Stable	Stable or transient	Stable	Stable or transient	Transient	Transient	Stable or transient

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Table 3. Types of Gene Vectors *(continued)*

	Viral							Nonviral
Vector localization	Nucleus	Nucleus	Nucleus	Nucleus	Nucleus	Cytoplasm	Cytoplasm	Nucleus
Types of cells transduced	Dividing only	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent
Efficiency of gene transfer	High	High	High	High	High	High	High	Low
Expression of viral proteins	No	No	Yes, unless viral genes deleted	No	Yes	Yes	Yes	No
Other	Tropism can be altered by pseudotyping		—	—	—	Can be used as a plasmid therapy system	—	—

VECTOR DESIGN CRITERIA

Many types of gene therapy vectors are being developed, and the vector selected for a particular clinical application depends on the disease state, the target cell, and the intended route of administration. As shown in *Table 3*, capacity depends on vector type, so clinical applications that require a large amount of genetic material will limit the choice of vector system. The payload of a vector system becomes increasingly important when one designs vectors with genomic DNA or a vector that contains extensive regulatory sequences.

Vectors are also selected based on the intended duration of expression and the target cell. For example, retroviral vectors integrate stably into target cells and are therefore well suited for stem cells or lymphocytes that are expected to undergo extensive cell division. In contrast, adenoviral and plasmid vectors are episomal and may be lost during cell division. However, adenoviral vectors are attractive for vaccine development and cancer applications where tumor cell elimination is the goal. Other vectors, such as AAV, do not integrate at high efficiency but can be expressed long-term in nondividing cells such as neurons or hepatocytes.

Target cell type can also play into the selection of an appropriate vector system (see *Targeting Transduction*). For example, the ▲▲ (USP 1-May-2020) Coxsackie virus B and adenovirus receptor (CAR) is expressed poorly on hematopoietic tissues, which limits the usefulness of the vector system for blood-derived cells. Vectors based on murine retroviruses require cell cycling and are not well suited to the transduction of nondividing cells such as neurons.

The immune system can target both the viral components of the vector and the expressed transgene. Pre-existing antibodies or cellular immunity to certain vector systems can exist and may limit their usefulness. Vectors can elicit an innate immune response that can decrease the efficiency of gene transfer and may also induce a severe adverse event. A large number of current gene therapy approaches seek to limit toxicity and immune response by administration of vector to cells ex vivo. Nevertheless, the majority of diseases suitable for gene therapy will require in vivo administration, and ongoing research seeks vectors with limited immune recognition.

The route of administration and manipulation of the total dose of vector are strategies that can be used to compensate for some limitations of specific vector systems. Additionally, there are advantages and disadvantages for the manufacture of each of the different vector systems that should be considered when planning a clinical application. Production consistency favors systems with well-defined fermentation or culture systems, such as plasmid, retroviral, or adenoviral vectors. For viral vector systems that require helper functions (see below), a rationally engineered cell line can overcome the scalability and consistency limitations of co-transfections. Use of a cell line that is adapted to suspension culture can affect scalability and cost efficiency.

TARGETING TRANSDUCTION

To be effective, a vector must first find and transduce its target cell. Viruses have a natural host range that is strongly influenced by the expression of specific cell-surface receptors, the current phase of the cell cycle, and the route of administration. Integrins are a class of cell-adhesion receptors that interact with either the penton base or the fiber protein of adenoviruses. The fiber and penton base proteins of adenoviruses mediate binding to the CAR, CD46, and integrins. Adeno-associated viruses primarily interact with heparan sulfate proteoglycan and sialic acid receptors on the cell surface. However, interaction with secondary receptors such as integrins, laminin, and growth factor receptors is required for efficient cell entry and trafficking of virus particles to the nucleus. An amphotropic variant of the murine leukemia virus (MLV), commonly used for gene therapy applications, utilizes the sodium-dependent phosphate transporter RAM-1 to enter cellular targets. Expression levels of each of these receptors vary according to tissue type, which dictates the transduction efficiency of the vector.

The host and tissue range can be modified or targeted by biochemical and genetic manipulation of the vector. Alterations in the tissue and cell specificity of retroviruses—and lentiviruses in particular—occur largely through genetic pseudotyping. During this process, the envelope proteins that dictate virus binding and entry via a specific cellular receptor of one virus are replaced with the envelope protein of another retrovirus or with a protein from an entirely different virus such as the vesicular stomatitis virus glycoprotein. The relative complexity of adenovirus and adeno-associated virus capsids allows them to be genetically modified in several ways. Substitution of a single virus protein (e.g., adenovirus fiber or AAV VP1) with that of another serotype within the same family is very much like the pseudotyping process for retroviruses. However, mosaic virus particles created by interspersing individual capsid proteins from several different virus serotypes in one virion, and chimeric particles created by capsid proteins of two distinctly different serotypes on a particle of yet another serotype (e.g., adenovirus 35 knob on adenovirus 41 fiber on an adenovirus 5 particle) can also effectively change the types of cells and tissues that a vector can transduce. Although this approach may seem straightforward, certain modifications of virus capsid proteins at the genetic level do not facilitate virus particle formation. Although most modifications improve virus uptake in one specific cellular target, they

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may also increase uptake in several other tissues in which gene transfer would not be desirable. Thus, selection of proteins and ligands must be carefully considered and tested in preclinical models of disease before these vectors can be used extensively.

Viral coat proteins and nonviral vectors can be chemically modified for ligand-mediated receptor targeting. They can be conjugated to cell-targeting ligands by antibody–virus interactions with bi-specific antibodies. Molecular bridges like biotin–avidin complexes and chemical crosslinkers such as bifunctional polyethylene glycol (PEG) tethered to cell specific receptor-binding proteins are easily conjugated to virus particles and often are incorporated in targeting strategies. These approaches can easily be combined and/or exchanged with genetic modifications and with each other to create vectors that effectively target several receptors. Although the biochemical approach avoids the functional complications of introducing foreign domains into viral proteins, each lot of vector must be modified because progeny virus will default to their original genetically encoded tropism. Biochemical processes also require the use of multiple reagents, which may complicate transfer to the clinic.

Another common and effective strategy to target viral vectors to tumor cells takes advantage of the virus replication cycle. Deletion of genes critical for taking over functional cellular checkpoints to support normal virus replication allows the virus to replicate only in cancerous cells where those checkpoints are either defective or inactive. This effect can be further enhanced creating small mutations in virus replication genes driven by tumor- and tissue-specific promoters. With respect to cell cycling, adenoviruses and adeno-associated viruses easily infect both quiescent and dividing cells, but MLV-based retroviral vectors are efficient only when transducing rapidly dividing cells. Lentiviral vectors can infect quiescent cells, including cells of neuronal origin. In general, nonviral vectors can enter both dividing and nondividing cells but have lower transduction efficiencies than viral vectors. Transduction efficiencies of nonviral vectors can be enhanced by the formulation and direct injection in the tissue of interest.

INFLUENCE OF HUMORAL IMMUNE SYSTEM AND COMPLEMENT

One of the most significant barriers to effective gene transfer is the humoral immune response to the vector. Regardless of the route of administration, the intended target cell, and the dose, the vector is likely to encounter some component of the immune system. For viral vectors, the humoral immune system cannot readily distinguish between wild-type viral infections and recombinant viral vectors because the humoral response is directed against proteins in the viral envelope or capsid. Protein-containing formulations of nonviral vectors can also elicit a humoral immune response. Specific and cross-reacting humoral responses may pre-exist because of natural exposure to wild-type versions of viral vectors, or they may be elicited during dosing, and the antibody response may vary in its capacity to diminish gene transduction in individual patients. Because neutralizing capacity is frequently enhanced upon multiple dosing, repeated administration can also be problematic. Some of these issues may be remedied by the use of nonviral systems. However, the level of transduction efficiency of these vectors is not currently sufficient for many gene-transfer applications. Transduction efficiency of all vectors is also importantly compromised by the complement system. Many complement proteins have a natural affinity for virus capsid proteins. This interaction initiates release of cytokines and chemokines that facilitate rapid removal of vector from the systemic circulation. This affinity is often heightened (especially in the case of retroviruses) when nonhuman cellular proteins and culture components (e.g., fetal bovine serum) are incorporated into and/or coat the virus particle during large-scale production. Use of producer cell lines of human origin and serum-free culture conditions has decreased inactivation of vectors by complement.

Several strategies for mitigating and avoiding the humoral immune response and complement inactivation have been developed. Increasing the vector dose to compensate for the neutralizing activity of the antibodies and altering dosing regimens to coincide with periods of low antibody titer are logical choices, but possible toxicity associated with high doses of virus and the individual variability of the immune response are potential negative consequences of this approach. Alternatively, viral vectors can be engineered to evade the immune system. One approach involves increasing expression of specific viral genes that allow the virus to evade the host's humoral response. Recombinant viruses constructed from serotypes with limited exposure rates such as simian adenovirus 7 can avoid neutralization in those previously exposed to more common serotypes. Mosaic and chimeric viruses can also avoid neutralization. Both approaches effectively address the issue of efficient gene transfer in those with pre-existing immunity, but both approaches require further investigation in response to concerns regarding safety and large-scale production as well as induction of immune responses in naive patient populations. Covalent attachment of polyethylene glycol (PEGylation) to the virus capsid can both protect the virus from neutralization and blunt the immune response. Pharmaceutical methods such as embedding viral vectors in polymer matrices and administration of vectors to the mucosa (oral and nasal) can also protect viral vectors from the humoral immune response.

INFLUENCE OF CELLULAR IMMUNE RESPONSES

Once transgene expression is initiated, cellular immune responses rapidly remove cells transduced by both viral and nonviral vectors. This decreases the overall therapeutic effectiveness of gene transfer of low-to-moderate vector doses and can be highly toxic when higher doses are administered. Active protein synthesis is not required for cellular immune responses to viral capsid proteins. For example, the capsid proteins of recombinant AAV vectors have been shown to be long-lived, leading to a delayed immune response and elimination of vector-transduced cells. De novo synthesis of viral gene products can also exacerbate host-cellular responses. Viral vectors have been designed with specific backbone deletions to eliminate the expression of viral structural genes and reduce this effect. Examples of such vectors include gutless adenoviruses, herpes viruses, and adeno-associated viruses in which all viral genes have been deleted, making them dependent on another helper virus for subsequent replication. Certain plasmid sequences, especially those with unmethylated CpG dinucleotides, can elicit a strong cellular immune response and have been used as adjuvants in some DNA-based vaccines. Amplifying plasmids in bacteria that express the CpG Methylase (M.SssI), removal of CpG sequences by site-directed mutagenesis, and removal of unnecessary prokaryotic sequences to create minimal plasmids have reduced the incidence of unwanted cellular responses. One nonmolecular approach to minimize the cellular response against the vector involves the use of immunosuppressants (cyclosporine, sirolimus, dacluzumab) at the time of initial vector administration as well as methods described for reducing the humoral response (use of chimeric vectors, PEGylation) outlined above.

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ANTIGENICITY OF THE GENE THERAPY PRODUCT

In many cases, the gene therapy product and associated promoter and enhancer elements are antigenic in certain cellular targets. When proteins that are retained in the target cell are used, cellular responses may eliminate the target cell. If sustained protein expression is required, the cellular immune response may decrease the effectiveness of the therapy or eliminate it entirely. In terms of treating genetic diseases, patients with a null mutation who have never seen the transgene product may be at a higher risk for immune response than patients who produce a defective protein. Also, truncation of a gene such as the cystic fibrosis transmembrane conductance regulator (CFTR) so that it fits within a chosen vector may result in creation of a distinct antigen.

In other applications, the transgene product is a foreign protein, e.g., thymidine kinase derived from the herpes simplex virus (HSV), and thus may elicit an immune response. In some cases this is the desired therapeutic effect, particularly in antigen-based immunotherapy for cancer or a viral disease. Efforts to minimize the immune response against elements associated with the transgene cassette include designing vectors with the ability to carry full-length humanized sequences for the transgene of interest and administration of immunosuppressants at initial dosing and other intervals throughout the treatment protocol.

VECTOR LOCALIZATION WITHIN THE TARGET CELL

Once the vector reaches the target cell, several factors can affect the level and duration of therapeutic gene expression, and these factors dictate the choice of an appropriate vector system for a specific clinical indication. The localization of the vector genome within the cell, the strength of the gene expression control elements, the stability of the message, and the stability of the translated protein all affect therapeutic impact. Alphavirus-based vectors, such as those derived from Sindbis or Semliki Forest virus, reside in the cytoplasm and typically exhibit a very high level of gene expression. Retroviral, adenoviral, and other viral vectors have advantages in gene delivery with their natural mechanisms for nuclear delivery of the therapeutic gene and reasonable levels of gene expression from viral or other promoters. Nonviral plasmid vectors are episomal and are often susceptible to DNA degradation when they are shunted into cell endosomes. However, some nonviral systems incorporate nuclear targeting signals as a means of increasing therapeutic gene transcription efficiency.

TISSUE-SPECIFIC EXPRESSION

Another means of controlling gene expression is the incorporation of tissue-specific promoters to stimulate or to restrict expression of the therapeutic gene. Unfortunately, many tissue-specific promoters do not provide high levels of gene expression, and incorporating these sequences into viral vectors may result in loss of specificity or low-level expression in cells that do not normally express the promoter. Tagging vector with sequences recognized by the microRNA system has also permitted tissue-specific expression and may offer tighter control than typically seen with tissue-specific promoter systems.

Drug-responsive promoters are being used to control gene expression. Rapamycin, mifepristone, and tetracycline (tet-on) systems have been used to repress gene expression. This type of regulation is particularly useful when constitutive expression of the vector transgene is toxic.

IMPACT OF REPLICATION STATUS OF VECTOR

Replication status is another important consideration for vector design and selection. Viral vectors are most frequently constructed to be incompetent or replication-defective in order to limit uncontrolled vector spread and pathogenicity. However, the ability to replicate and spread within a specific cell population, e.g., within a tumor or to metastatic sites, may provide a significant therapeutic advantage over cell type-specific targeting of replication-incompetent vectors. Replication can be engineered to be conditional when, for example, specific viral gene interactions are matched with intracellular pathway targets by means of targeted deletions and/or changes in transcriptional or translational control. When these targets are defective or missing, as in cancer cells, the virus can replicate, but when the target cell is functioning normally, viral replication is repressed. Viruses that have been genetically engineered for selective oncolytic replication include: adenovirus, HSV, vaccinia, measles virus, picornaviruses, influenza virus, Coxsackie virus, and Sendai virus. Some nongenetically-modified viruses are inherently oncolytic in human cells, e.g., reovirus and Newcastle disease virus.

One of the risks inherent in the use of conditionally replicating viral vectors is that such systems are leaky, i.e., the growth of the virus is not absolutely restricted to a single cell type. Also, subsequent rounds of viremia become considerations in the evaluation of tissue distribution/exposure and shedding. The therapeutic promise of these approaches depends on the reliability with which conditionality of replication can be selected or engineered. This therapeutic potential will be realized only if balanced with steps to control the potential risks to patients (associated with replication competence of the viruses/viral vectors) and to address associated shedding-related issues of third party exposure and environmental concerns.

As a proactive contribution to the safety profile and to take advantage of scientific and clinical information already available, virus strains that have been used for human vaccination are often used as the vector backbone. Nevertheless, because the product is replication competent, it presents specific technical challenges for adventitious agent testing and product characterization.

Nonviral vectors are normally designed as nonreplicating systems, but some groups are experimenting with replicating nonviral plasmids to increase gene expression levels (because of the low transduction efficiency of most nonviral systems) and to increase the duration of gene expression. Additional preclinical studies are needed to establish the safety of these systems. Artificial chromosomes have also been designed to take advantage of normal mechanisms for retaining gene expression in rapidly dividing target cells.

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VECTOR INTEGRATION

The duration of gene expression is also a function of the persistence of the vector genome in target cells. Retroviral vectors can stably integrate into the host-cell genome, providing long-term expression. Adenoviruses and nonviral plasmid vectors, e.g., those not administered using electroporation, do not integrate, and expression generally decreases over time. Recombinant AAV vectors generally do not integrate, and when they do, it is not site-specific. However, stable episomes have been observed in certain cell types such as muscle cells.

Site-specific integration can be a desirable feature for vectors that are intended to correct genetic disorders. Although it is not currently efficient enough to be useful, the control of the site of integration is desirable in order to prevent insertional mutagenesis. Insertional mutagenesis has the potential to kill a cell if a critically functioning gene is inactivated or to predispose a cell to malignant transformation if a tumor-suppressor gene is inactivated. Of clinical relevance, promoter or enhancer elements within vectors can lead to activation of cellular oncogenes and have been associated with malignant transformation in children undergoing retroviral gene transfer for X-linked severe combined immunodeficiency.

The success of any gene therapy product depends on the relationship between the vector-delivery system and the requirements of the disease in terms of the site, level, and duration of therapeutic gene expression. A universal vector now appears unlikely, and the challenge lies in fitting one of several possible vectors to the disease and to the gene to be delivered.

Manufacturing and Purification Strategies

VECTOR CONSTRUCTION

Viral and nonviral gene-transfer vectors are constructed by using standard molecular biology protocols. For viral vectors, the vector backbone consists of viral RNA or DNA sequences from which the regions encoding viral structural genes or the regions required for replication have been deleted. The deleted region of the vector is usually modified with specific restriction endonuclease sites used to allow insertion of the gene of interest. For nonviral vectors, the plasmid DNA backbone contains multiple restriction sites for cloning and the bacterial elements necessary for plasmid production. Vector backbones can accommodate single or multiple gene insertions depending on the maximum amount of sequence they can carry. The promoter that facilitates transcription of the gene insert can be a related viral promoter, such as the murine leukemia virus long terminal repeat (MuLV LTR), or a heterologous promoter that is either tissue-specific, such as the alpha crystalline promoter (of the eye), or constitutive, such as the cytomegalovirus (CMV) late gene promoter. For example, in a retroviral vector construct containing two gene inserts, transcription of one is regulated from the 5′-LTR-promoter sequence, and a second gene insert can be linked to an internal heterologous promoter from Simian virus 40 (SV40).

The complementary DNA (cDNA) containing the therapeutic gene of interest, including its introns, is excised from its source using restriction enzymes and is inserted at the multiple cloning site of the gene-transfer vector. The polyadenylation signal can be derived from multiple sources such as the SV40 virus or human growth hormone gene. Characterization and testing of gene therapy vectors are described under *Analytical Methods*.

HELPER FUNCTION SYSTEMS

Recombinant viral vectors are most often modified to be replication defective, a condition created by deletion or modification of the viral genes needed for replication and production of infectious virus. Because the vectors are stripped of some or all of the viral genes, a system must be developed to supply viral proteins and to encapsulate the vector into a viral particle. Generally, this is accomplished by two methods: transient transfection or stable packaging cell lines.

In the transient transfection method, a series of different plasmids are generated, including a plasmid containing the vector and another vector containing the viral genes. For example, retroviral vectors can be generated using a three-plasmid system: 1) the transgene-containing vector plasmid; 2) a plasmid containing the *gag/pol* viral gene region; and 3) a plasmid containing the viral envelope. All three plasmids are transfected into cells, e.g., HEK293 or HT1080, and vector-containing virions are harvested after 2–3 days. The separation of vector and viral genes on different plasmids, along with vector designs that minimize the homology between vector and viral sequences, decrease the chance for recombination and generation of replication-competent virus. Similar approaches can be taken with most viral vector systems.

The transient transfection method has the advantage of a rapid production time and flexibility when changing components of the vector or viral constructs. Nevertheless, it can be cumbersome when scaling up for manufacturing, and special care must be taken to provide consistent production yields. An alternative method has been the use of vector packaging cell lines. In this scenario, the viral genes are introduced stably into an immortalized cell line that yields persistent expression of viral genes. As with transient transfection, the viral genes generally are expressed from different plasmids to decrease the risk of recombination. Since plasmids integrate infrequently, considerable time and effort are required to isolate a high-titer packaging cell line and to generate an MCB. Vector constructs can be introduced into cells from the MCB, and researchers, by screening for a high-titer clone may allow isolation of a stable cell line that generates the vector of interest. These cell lines generally can be expanded to great numbers and often produce vector for up to a week at a time, facilitating vector scale-up and product consistency.

Typical helper function systems are as follows:

Retroviral vector systems: Initial packaging cells were based on the murine fibroblast cell line NIH 3T3. The PG13 cell line (expressing the Gibbon Ape Leukemia Virus envelope) has been used extensively with a low incidence of recombination events leading to RCV. More recently, the human HEK293 and HT1080 cell lines have been modified to serve as packaging cell lines for retroviruses. The use of a human cell line decreases elimination of vector particles by the human complement system (although this is generally not a concern for vectors used in ex vivo protocols).

Adenoviral vector systems: HEK293 cells are widely used to supply the E1 function necessary for efficient adenoviral replication that is deleted from first-generation adenoviral vectors. Other complementing cell lines, such as E1-modified A549 cells (human lung carcinoma) and the PER.C6 cell line (human embryonic retinoblast), have also been created to supply E1 or other missing

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functions. PER.C6 contains the E1 region under the control of a phosphoglycerate kinase (PGK) promoter and has no flanking adenoviral sequences in order to eliminate production of replication-competent adenovirus (RCA).

AAV vector systems: These systems classically use adenovirus-infected HEK293 cell lines transiently transfected with AAV helper plasmid containing the *rep* and *cap* genes, which are required for AAV replication and capsid formation, respectively, and are deleted from the AAV vector. In some AAV production systems, wild-type adenovirus has been removed from the process by using triple transfection of plasmids expressing *Ad* early genes, *rep* and *cap*, and the vector transgene. The HeLa cell line (from human uterine cervical carcinoma) has also been used as a transient production system. More recently, both of these cell lines have been used to establish stably transfected packaging cell lines that express *rep* and *cap* genes and in some cases express the adenoviral functions needed for AAV replication when *rep* and *cap* are present (E1a, E1b, E2a, E4, and VA RNA). AAV production systems using recombinant HSV and Baculovirus have also been developed.

Gutless adenoviruses: Early manufacturing systems for the adenovirus vector known as gutless adenovirus were similar to classical AAV vector manufacturing systems because HEK293 cells were transiently transfected with helper plasmid containing required adenoviral functions. Development of helper viruses housing a packaging signal flanked by loxP sites and complementing HEK293 cell lines that express the bacteriophage P1 site-specific Cre recombinase has greatly improved the yield of the gutless virus. This technology notably reduces the amount of helper virus contamination by preventing packaging of the helper virus genome while permitting it to replicate and support replication and encapsidation of the gutless vector.

VIRAL GENE THERAPY VECTORS

Retrovirus and adenovirus vectors typically have been produced at laboratory, non-GMP scale by use of traditional cultivation methods for anchorage- and serum-dependent cell lines employing flasks, trays, and roller bottles. Initially, gene therapy vectors were produced by these methods because large volumes of product were not required for early clinical studies. Cell-bank systems are used as the source of cells, and virus banks are the source of virus for clinical production. In many cases, supernatant is collected, clarified, and stored frozen in bags at −70°. In many early clinical trials, unpurified supernatant has been used for ex vivo gene transfer.

Larger-scale upstream production methods have been reported and are commonly used. They include suspension, bioreactor, and fixed-bed or microcarrier culture methods. Some groups have reported adapting their process cells to serum-free culture conditions. Cells are harvested and lysed or supernatant is collected. The harvest is clarified and purified to remove host-cell debris, host-cell DNA, and other process-derived contaminants.

Traditionally, viruses are purified by gradient ultracentrifugation, but this is time-consuming and unsuitable for larger-scale production purposes. The selection of downstream process steps and their sequence is determined by the nature of the virus itself and the upstream process used for manufacturing the virus. As processes are being developed for the manufacture of gene therapy vectors, many different purification steps have been reported. These include ion-exchange and sulfonated-cellulose chromatography, zinc ion affinity chromatography, and size-exclusion chromatography. Typically, DNase or other nuclease treatments are used in the process in order to reduce host-cell or plasmid DNA. AAV production and lentiviral production are complicated by a need for transient transfection or co-transfection of plasmid or helper virus. These processes have generally required anchorage-dependent cell lines that are difficult to scale up. The development of stably transfected cell lines would allow large-scale production.

PURIFICATION METHODS: VIRAL VECTORS

Retroviruses: To date, purification of retrovirus preparations for Phase I clinical trials has often been minimal at best, i.e., the simple concentration of culture supernatants is insufficient to meet the stringent quality standards required for in vivo therapy. Centrifugation and microfiltration techniques are very useful for clarification of culture supernatants and removal of cellular debris. Ion-exchange, size-exclusion, and affinity chromatography techniques have also been employed to remove excess salt, serum, and low molecular weight contaminants also concentrated with the virus.

Adenoviruses: Recombinant adenoviral vectors were often purified by cesium chloride density gradient ultracentrifugation. This is still used for research-scale preparations, but the procedure is neither scalable nor efficient for large quantities of clinical-grade virus. The most recent scalable purification methods use anion-exchange chromatography because of the strong affinity of intact virus particles for the resin with respect to that of cellular and individual capsid proteins. Published loading estimates for anion-exchange resins range from 0.5×10^{12} to 5×10^{12} virus particles/mL of resin or 0.14 mg of virus to 1.4 mg of virus/mL of resin. Gel filtration and immobilized metal affinity chromatography are often used in polishing steps following anion-exchange purification of recombinant adenovirus-based products.

Adeno-associated viruses: The toxicity of cesium chloride, the aggregation of AAV particles, and the fact that adenovirus is not completely removed after extensive centrifugation complicate AAV purification by cesium chloride density gradient ultracentrifugation. Another density separation medium, iodixanol, which is less toxic than cesium chloride and prevents AAV aggregation, has been employed in a single centrifugation step. Passage of the AAV fraction over an affinity column consisting of either a heparinized support matrix or monoclonal antibodies produced against AAV2 strongly increased purity and infectivity of final preparations. These methods are appropriate only for specific AAV serotypes. Ion-exchange chromatography is the most powerful and versatile method for AAV purification, although buffer pH, detergent concentration, and column medium must be tailored for each AAV serotype. Infectivity and purity of preparations obtained from these purification strategies are comparable to those obtained from affinity chromatographic methods and are complete within 3 h.

PLASMID OR NONVIRAL VECTORS

Plasmids are double-stranded, circular DNA molecules that exist in bacteria as extrachromosomal, self-replicating molecules. They have been modified to serve as cloning systems, to contain multiple restriction endonuclease recognition sites for insertion of the cloned transgene, and to contain selectable genetic markers for identification of cells that carry the recombinant vector. Plasmid-based, nonviral vectors are frequently used as gene delivery systems for both in vivo and ex vivo gene therapies. These

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vectors are in the form of naked DNA or are complexed with lipids or other agents that facilitate transfer across the cell membrane and delivery to the cell nucleus without degradation. An advantage of a plasmid-vector system is the efficient production of large quantities of the vector that is easily characterized and avoids the risk of RCV associated with many viral systems.

Nonviral vectors are typically produced by using an *Escherichia coli* bacterial system. Plasmids are transfected into *E. coli*, and an appropriate single bacterial colony is selected and expanded to create an MCB. After reselection of a colony from a bacterial plate inoculated from the MCB, plasmid DNA is isolated from cultures that can range in size from 1 L on a laboratory scale to hundreds of liters in bacterial fermenters. Plasmid DNA can be purified by several methods including affinity or ion-exchange chromatography and cesium chloride–ethidium bromide density gradients. Cesium chloride–ethidium bromide density gradients are not recommended for production of clinical-grade material.

PRODUCTION AND PROCESSING OF NONVIRAL VECTORS

One benefit of nonviral vectors for gene transfer is that the production process is rather generic and can be applied to any plasmid preparation regardless of composition or application. Because the current average human dose of plasmid DNA for gene transfer and vaccination is approximately 1 mg, the primary challenge associated with large-scale production of plasmids is to develop a process that is both scalable and economical. Thus, process development for plasmid-based vectors remains an active area of research and development. A standard process for large-scale production of recombinant DNA plasmids consists of the following five unit operations.

Fermentation: Fermentation processes must support growth of transformed bacteria and maximize the amount of plasmid produced by each cell. *E. coli* is the most common strain used for plasmid production. Amino acids, nucleosides, and the ratio of nitrogen to carbon-containing compounds present in a rich media formulation greatly improve plasmid yield.

Harvest: Bacterial cells are harvested either by centrifugation or microfiltration. Centrifugation under GMP conditions can be costly, which makes microfiltration the accepted method of cell harvest. This also allows spent media, metabolic byproducts, extracellular debris, and impurities to be washed away before purification.

Lysis: Bacterial cells must be lysed to release the recombinant plasmids. This is one of the most critical steps in the production process because it can significantly affect the amount of usable [covalently closed circular (ccc)] and unusable [sheared, partially denatured, and open circular (oc)] forms of DNA in a preparation. The most widely used method of lysis for clinical-scale manufacturing is treatment with alkaline detergent and precipitation of cellular debris with acetate. This removes a large fraction of cellular impurities from the lysate, but it also increases the sensitivity of plasmids to mixing and localized concentrations of detergent, which are hard to manipulate on a large scale. Lysis of cells by heat exposure addresses this issue and effectively denatures cellular proteins and bacterial DNA.

Isolation/purification: Some processes include additional steps for removal of cellular debris and other contaminants from crude bacterial lysates by precipitation with detergents, polyethylene glycol, or salt. These reagents affect plasmid stability and are removed by column chromatography. Size-exclusion chromatography can effectively separate plasmid DNA from RNA, proteins, and other small molecules present in the cleared lysate. The degree of separation of plasmid DNA from contaminants is highly dependent on the type and concentration of salt in the running buffer. Resins used in anion-exchange chromatography have a high affinity for plasmid DNA and provide maximal sample concentration. Hydrophobic interaction and thiophilic aromatic chromatography are the methods of choice for selective separation of the different plasmid DNA isoforms and endotoxin reduction.

Bulk preparation: After purification, the bulk plasmid is placed in a suitable buffer and formulation by ultrafiltration using a membrane with a pore size of 50–100 kDa.

Plasmids for clinical use must be highly characterized. Impurities from production and processing steps are well known. Tests necessary to confirm the identity, purity, and potency of a plasmid-based product are well established and routine. These tests and the current specifications set by the FDA and the World Health Organization are summarized in *Table 4*.

Table 4

Assay Type	Issue	Determined By	Acceptable Level in Final Product
Identity	Cross-contamination with other products	Restriction digest/gel electrophoresis	N/A
Purity	Residual bacterial chromosomal DNA	Real-time polymerase chain reaction (PCR)	<2 µg/mg DNA
	Residual RNA	Analytical HPLC	<0.2 µg/mg DNA
	Residual bacterial protein	Bicinchoninic acid (BCA) protein assay	<3 µg/mg DNA
	Endotoxin	Limulus amebocyte lysate (LAL) assay	▲Suitable criteria based on the final manufacturing process▲ (USP 1-May-2020)
	Sterility (bacterial and fungal)	Method outlined in 21 CFR 610.12	No growth
	Appearance	Visual inspection	Clear solution free of particulates
	pH	pH meter	Physiologic (7.0–7.4) but may be product-specific
	Plasmid confirmation (ccc vs oc)	HPLC or capillary gel electrophoresis (CGE)	>97% ccc

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Table 4 *(continued)*

Assay Type	Issue	Determined By	Acceptable Level in Final Product
Potency	Labeled dose	In vitro Enzyme-linked immunosorbent assay (ELISA) Fluorescence-activated cell sorter (FACS) Reverse transcription (RT)-PCR Light absorbance (A_{260})	Transgene/plasmid specific

Introduction of Genetic Material into Cells—Gene-Modified Cells

A common extension of cell therapy involves the introduction of genetic material, usually DNA, into cells to alter their pattern of gene expression. While discussion focuses on DNA, similar scenarios can be applied to RNA or a derivative of DNA, except that the stability and solubility of the particular nucleic acid may dictate modifications of certain steps. The general process is often referred to as ex vivo gene therapy because the cells are removed from the patient or donor and the genetic material is introduced while the cells are outside of the body. The genetically modified cells are then administered to the patient. The genetic material introduced can either cause expression of new genes and products or inhibit the expression of already expressed genes and products. The latter represents a type of antisense therapy. The genetic material can be introduced by the same range of reagents that are involved with gene therapy: viral vectors, nucleic acids in a simple formulation (naked DNA), or nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Most of the steps and considerations discussed above also apply to the ex vivo introduction of genetic material into cells. The main goal of ex vivo therapy is to develop robust processes that will work with the majority of the patient's or donor's cells. This takes considerably more effort than processes for cell lines.

The method for introducing new genetic material into cells depends on the biology of the system and the desired stability of gene expression. If a simple retroviral vector such as ▲Moloney▲ (USP 1-May-2020) murine leukemia virus is used for transduction, the cells must be actively dividing because vector DNA is integrated into the cellular DNA only during replication. This usually leads to long-lasting expression of the desired gene product. Adenoviral vectors, naked DNA, or formulated DNA can be introduced into nondividing cells. However, gene expression will be transient because the introduced DNA will usually be extrachromosomal.

The main challenge in ex vivo gene therapy is to achieve efficient transduction or transfection, introducing sufficient DNA into the cell before the DNA degrades. In the case of transduction by simple retroviral vectors, cells are stimulated with reagents that cycle them into the S phase (replication) at the time that the vector is applied. Most retroviral vectors are stable in cell culture for a period up to a few hours. Because diffusion is minimal, only a small fraction of viral particles will come into contact with cells during this period. The following techniques can be used to increase the number of viral particles that contact the cell in a given time period:

1. Maximization of viral particle concentration and minimization of the media volume during the transduction step.
2. Multiple applications of the virus.
3. Centrifugation of virus particles onto the cells.
4. Placing cells on a filter and slow pulling viral media through the filter.
5. Addition of binding-enhancing polymers to the media. [NOTE—Co-culturing the target cells with the viral producer cells is not recommended. This technique increases the chance of a recombinant event and production of RCV. Furthermore, any product for which co-culturing is used to transduce the human cells would be considered a xenotransplant if the producer cells were not human. The second cell type, whether human or not, may cause inflammation.]

Each of these techniques has its own set of issues that must be addressed in order to develop a robust process. In technique 1, reduction of the volume during transduction results in rapid exhaustion of the medium, so supplemental medium should be added within a few hours. In technique 2, the cells may no longer be in the correct cell cycle phase during later applications, or cells may have become refractory because of unproductive transformation during the previous application. Techniques 3 and 4 can work well on a very small scale, but the number of cells that can be transduced may be insufficient to obtain an efficacious dose. In technique 5, polymers may fail to provide a benefit because virus binding may involve specific receptors for which surface density may prove to be the limiting factor.

Similar issues and techniques can apply with other viruses or DNA preparations. The issue of slow diffusion is even more marked for the use of DNA preparations. Factors such as the cell type in which the viral vector was produced, the media used for vector production, and the purity of the vector can have a pronounced effect on the efficiency of transduction. Although certain methods may not require cells to be actively cycling, in practice, most processes require that cells be capable of replication because of the following considerations:

1. Safety considerations may dictate that only cells that express the new DNA are returned to the patient, which requires that these cells be selected. As described below, the most common selection method uses an antibiotic-resistant gene that is co-introduced with the new genetic material.
2. Further propagation may be required to achieve the therapeutic dose of cells.
3. Economic, biological, or technical reasons may dictate that the DNA introduction step be carried out at a low cell number and that the desired cell population then be expanded to the required dose.

Therefore, conditions that enable the cell or maintain its ability to proliferate must be developed in almost all cases. The biology of the cells, the available technology, and process economics will determine whether cells are propagated before, after, or during the introduction of new genetic material. Most processes do in fact expand the population after the introduction of the new gene.

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Whether cells that do not productively express the gene can be administered to patients depends on the biology of the application, the dose required versus the handling capability of the manufacturing system, and, most importantly, the toxicity of the nonproductive cell population. Selection of the genetically modified cell population is commonly carried out using an antibiotic-resistance marker gene, such as neomycin, which is co-introduced into the cell with the new genetic material. For neomycin selection, cells in culture are treated with the antibiotic G418 at a concentration and for a period that has been shown to kill cells with nonproductive expression while allowing the productively expressing cells to proliferate. In this manner cells that are resistant to the antibiotic are presumed also to express the DNA of interest. The expression should be tested as a lot-release requirement or verified in a series of mock runs. Because most antibiotics decrease cellular proliferation, optimization of the culture media composition may be necessary for efficient selection and propagation of the gene-modified cells.

Following the antibiotic selection step, a second phase of antibiotic-free cell propagation may be required in order to achieve the desired dose and to rinse residual G418 out of the system. The selected medium and the total time that the cells are in culture can be critical to maintaining the desired expression of the original differentiated functions. An additional issue associated with the use of selection markers is that they generally are nonhuman genes. The expression of these genes usually elicits an immune response. Process development is often carried out with cells from healthy donors. Consideration should be given to the fact that for very sick patients, healthy cells that can be stimulated to undergo efficient, sustained replication are difficult to obtain.

Manufacturing, cell processing, and analytical testing issues pertinent to cell-based products are addressed in ⟨1046⟩.

Formulation of Gene Therapy Products

Final formulations for gene therapy products are still in early development, and currently most gene transfer vectors are stored in solution at ultra-low temperatures. Successful formulation of candidates for gene transfer relies on a thorough understanding of the physicochemical and biological characteristics unique to each vector system. Factors like solution pH, ionic strength, and osmolarity influence the thermal stability of viral and nonviral vectors. Organic carbohydrates such as mannitol, sorbitol, sucrose, and trehalose have been incorporated into preparations to prevent disruption of the native conformation of the vector in solution, during the freeze-thaw process, and during lyophilization. Amino acids such as arginine and leucine have been incorporated into formulations for their buffering effects and to prevent aggregation. Surfactants such as the Tweens, Spans, and Pluronics have been effective at preventing aggregation, but this effect is somewhat vector-specific because some vector products are easily disrupted by these reagents. Lipids, polymer, and extraneous proteins (human albumin and gelatin) have also been incorporated in many vector preparations because of their ability to prevent loss of vector from direct interaction with pharmaceutical surfaces and during freeze-thaw cycles.

Before initiating a program for formal screening of formulations for a vector, the following factors should be considered. The required dose and/or storage concentration of the final product, as well as the specifics of the container–closure and/or delivery system should be established. Analytical methods to assess potency and identify degradants should be in place. The expectations of the formulation must also be defined. Some pragmatic criteria for the design and selection of vaccine formulations for use worldwide are: 1) the final product should be in a formulation that affords an 18- to 36-month shelf life when stored at 2°–8° or above, 2) the formulation should have an acceptable stability profile at ambient temperatures to cover short-term storage and transportation in the field, 3) it should adequately protect the vector from damage during freeze-thaw cycling, and 4) it should consist of reagents that are pharmaceutically acceptable and within physiologically acceptable concentrations. Formulation changes during clinical development must be supported with preclinical studies and stability data. Sufficient time should be built into plans to account for this.

To date, little work in the area of formulation development of retroviral vectors with additives approved for human use has been described. The most significant effort to develop stable formulations for gene transfer has been with recombinant adenoviruses. Recently, identification of the mechanisms by which recombinant adenoviruses degrade in solution led to the development of several liquid formulations that stabilize the virus for up to 18 months at 4°. Adeno-associated viruses are regarded as one of the most stable viral vectors. This virus has been documented to be stable for approximately 4 months in phosphate buffered saline at 4°. Addition of cryoprotectants and surfactants prevents aggregation of virus particles and extends the shelf life to 1 year. Lyophilized formulations of both adenovirus and AAV with shelf lives of several years at room temperature have also been described. Although nonviral vectors have been found to be generally robust in standard buffers at 4°, their stability may be extensively influenced by extraneous components included to promote gene targeting. Consider the nature of those components when developing stability protocols and strategies. ▲▲ (USP 1-May-2020)

Change to read:

ON-SITE PREPARATION AND ADMINISTRATION

One or more product modifications or preparative steps may be required before administration of the gene therapy product to the patient. These modifications or steps are frequently performed close to the time of administration, and, therefore, they are performed under conditions not under control of the original manufacturing facility. The nature of these modifications is dictated largely by characteristics of the product in relationship to the particular application. These include thawing, washing, or filtration to remove unwanted product manufacture-related materials and also include defined physical space with appropriate environmental controls, trained personnel, detailed standard operating procedures, and a comprehensive quality program.

The unique and irreplaceable nature of many gene therapy products, e.g., gene-modified cells, many of which have originated from an autologous or a selected allogeneic tissue source, creates special considerations for product manufacture, release, and administration. Issues pertinent to the administration of cell-based products are addressed in detail in ⟨1046⟩.

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On-Site Preparation

PRODUCT MANIPULATIONS

Before administration, on-site preparation of the gene therapy product may involve one or more manipulations, including the following:

- **Change in final container:** The manufactured product may have been stored or transported in one container but may require transfer to a different container for administration.
- **Change in physical state or temperature:** A product may require thawing from the frozen state or warming from the refrigerated state.
- **Change in solution or suspension:** A product may have to be dissolved, diluted, or suspended in a liquid.
- **Addition to biocompatible structural material:** A gene therapy product may need to be combined with living, natural, or synthetic structural tissue or matrix. Examples of matrix material include hollow fibers, fibrous sheets, gels, plugs, capsules, sponges, or granules.
- **Admixture or compounding with other nonstructural materials:** A product may require mixing or compounding with drugs, cytokines, biologics, or other nonstructural materials.
- **Filtration or washing:** Unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations may require washing or filtration steps.
- **Sampling:** Sampling of the final product immediately before administration may be required for certain clinical protocols.

FACILITY REQUIREMENTS

Facility requirements for performing on-site preparative steps or administration of gene therapy products depend on the nature of the products, their applications, and the manipulations required. ▲▲(USP 1-May-2020)

RELEASE OF FINAL PRODUCT

Gene therapy products that undergo on-site preparative steps or manipulations must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations will determine whether release requirements or critical specifications must be added to those required immediately after initial manufacture. Prerelease requirements usually include the following:

1. Physical inspection of the product, which typically includes measures to ensure appropriate product appearance with regard to color, turbidity, particulates or foreign matter, container integrity; product temperature; and accuracy and convenience of labeling
2. Review of process records
3. For patient-specific products, clerical checking of product labeling or records related to the identity of the intended recipient.

▲▲(USP 1-May-2020)

Administration to Patients

PRE-ADMINISTRATION REQUIREMENTS

Depending on the specific gene therapy application, trained patient-care staff must take steps to prepare the patient for product administration. These steps are aimed at ensuring that the product will provide the intended therapeutic outcome and at minimizing the risk of adverse effects. Issues pertinent to administration of cell-based products are addressed in ⟨1046⟩. Generally, a thorough re-evaluation of the patient's general condition and suitability for therapy must be performed close to the time of product administration. This evaluation usually includes a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, staff may obtain baseline physical or functional measurements, laboratory tests, or imaging studies relevant to the specific application. Examples include pulmonary function tests for a therapy aimed at improving lung function, measurement of blood levels of an enzyme that is the gene product in a gene therapy application, and nuclear imaging of organs before anticancer therapies.

A variety of patient interventions related to route of administration may be required before product administration. For therapies that require intravenous administration, patients with poor peripheral venous access may require placement of a central venous catheter. In applications where gene-modified cells or matrices combined with cells are implanted into the patient, the site of implantation may require preparation in the operating room. This may involve surgically opening the site, removing the degenerated or damaged tissue, trimming of the adjacent tissue to accommodate the implant, and excising the tissue from a second site to be used as an anchor or support for the implant. For instance, in the case of products for wound healing, it is critical that the site for grafting be free from infection and that it demonstrates a well-prepared wound bed. Where gene-modified cells are intended to repair cartilage defects, the site of damage needs to be prepared so that the cells can be applied to a water-tight compartment. For applications involving direct administration of the product into an organ system (for example, bronchioalveolar system) or vascular network (for example, coronary arteries), the patient may require endoscopic or surgical access to these sites.

In all cases, the need for adequate anesthesia and premedication must be carefully evaluated in conjunction with these steps before product administration. Pre-administration patient evaluation must also include assessment of concurrent therapies that may interact with the gene therapy product to modify its effects. Some therapies may be considered adjunctive to the gene therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly

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used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects on the efficacy of the gene therapy product.

PATIENT TREATMENT

Some gene therapy products are patient-specific because they are manufactured from a selected tissue source, such as autologous, selected allogeneic, or xenogeneic tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, including special attention to the correct identification of the patient and patient-specific product by at least two people immediately before administration. These issues are addressed in greater detail in ⟨1046⟩. Gene therapy products can be administered by a variety of routes. These include parenteral injection, inhalation, and gastrointestinal routes. Other possibilities include direct application of gene therapy products into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. Although parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities such as an aseptic operating theater or endoscopic suite. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

POST-ADMINISTRATION MONITORING OF PATIENT

There should be written policies and procedures for monitoring patient outcomes and managing reports of adverse events. Patient outcome assessment should include indicators that are likely to detect errors or problems related to the entire manufacturing process, with special attention to manipulations, storage, or transportation after the initial manufacture of the product. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients with suspected adverse effects and a system for reporting and evaluating adverse effects that may point to a potential defect in the administered product. Reporting procedures include providing details required for federal, state, or USP adverse-event reporting programs.

Follow-up and monitoring procedures should be implemented for patients who have received gene therapy vectors or ex vivo gene therapies. To the extent that it is relevant and that it can be assessed, vector or gene-modified cell biodistribution and persistence in vivo should be monitored. With direct administration of vectors, localization to the germ line may be an issue. Although preclinical studies can address this issue, useful information may be gained by patient monitoring. When a retroviral vector has been administered, patients should be monitored for replication-competent retrovirus (RCR) according to the FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors* (October 2000). This involves active monitoring during the first year and archiving of patient samples thereafter if RCR is not detected initially.

Database systems to collate and track patient-monitoring results are essential to management of this information. National registries or publication of data should be considered for establishing the collective safety of gene therapy.

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ANALYTICAL METHODS

The complexity and scope of gene therapy products are reflected in the wide range of analytical procedures and their methods that are used to assess product quality. Approved gene therapy products must comply with applicable sections of 21 CFR 211 and 610 to ensure their identity, dose, potency, purity, and safety. Specific guidance for the identification, development, and validation of analytical methodologies to support cell and virus bank characterization, final-product release, and stability studies is currently provided in FDA guidelines for gene therapy manufacturing and testing (see *Appendix*); in ⟨1225⟩; and in the ICH guidelines Q2(R1) and Q6B. Most product-specific analytical methods for gene therapy products have not been standardized. Even well-defined tests such as those described under *Sterility Tests* ⟨71⟩ may not be directly applicable to certain gene therapy products. For some gene therapy products, large quantities of clinical material may not be available during early clinical development. Some required tests (e.g., sterility) may require modification. Consultation with regulatory authorities is advised.

Table 5 provides an overview of product-specific testing parameters for the biological component and general methods or approaches used to satisfy the testing requirements for nonviral, viral, and gene-modified cellular gene therapy products. The analysis of gene therapy products relies heavily on biological assays, but it also uses methodologies developed for biotechnology-derived products. The intent of this section is to outline the types of methods and their specific applications with regard to product characterization, stability, and release testing. Process validation may alleviate the need for certain specific lot-release tests. Development of appropriate reference materials and standards for viral, nonviral, and gene-modified cellular gene therapy products should be a part of product development. Reference materials should be fully characterized in order to provide continuity between standards over time. In the case of gene-modified cellular gene therapy products, the reference material may be a surrogate tissue or simulated product. Reference materials are briefly addressed in the FDA's *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products.*

Gene-modified cellular gene therapy products may require a rapid-release approach if they have a limited shelf life (see ⟨1046⟩). The rapid-release approach is not usually applied to viral and nonviral gene therapy products because these products are sufficiently stable for completion of testing before release. Some formulated nonviral gene therapy products also have limited shelf lives. In such cases, the individual components are tested before release and the formulated complex is not tested. The formation and stability of the formulated nonviral gene therapy complex is established via validation studies during product development.

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As specified in CFR, product samples must be retained after product-release testing is completed. Retain additional samples if rapid-release strategies are employed so that product quality can be reassessed by alternative or traditional test methodologies, if necessary.

Table 5. Analytical Tests for Cell and Gene Therapy Biological Products

Test	Gene-Modified Cellular Gene Therapy Product	Gene Therapy Products	
		Viral	Nonviral
Identity of Biological Substance	• Surface marker determination • Species • Morphology • Bioassay • Biochemical marker	• Restriction enzyme map • PCR • Immunoassay for expressed gene • Sequencing	• Restriction enzyme map • PCR • Immunoassay for expressed gene • Sequencing
Dose	• Viable cell number • Enumeration of specific cell population • Total DNA • Total protein	• Particle number • Transducing units (DNA hybridization assay) • Total protein • HPLC assay using authenticated reference standard	• Plasmid–DNA weight • Formulated-complex weight HPLC or capillary electrophoresis assay using authenticated reference standard
Potency	• Viable cell number (cells intended for structural repair) • Bioassays o Colony-formation assay o Function of expressed gene o Induction of secondary effect [e.g., human leukocyte antigen (HLA) induction, secretion of cytokines, and up-regulation of surface marker]	• Function of expressed gene (induction of secondary effect and other bioassays)	• Function of expressed gene (induction of secondary effect and other bioassays)
Purity	• Percentage of viable cells • Percentage of transduced cells • Percentage of cells with specific surface marker • Process contaminants (e.g., serum)	• Residual host-cell DNA • Process contaminants (e.g., serum and cesium chloride) • Residual helper virus • Optical density ratio • Residual host-cell proteins • Viral protein profile (HPLC assay for defective or immature particles) • Residual RNA	• Percentage of specific physical form (e.g., percentage supercoiled) • Residual host-cell DNA • Residual RNA • Residual host-cell proteins • Residual solvents • Optical density ratio • Process contaminants (e.g., cesium chloride)
Safety	• Mycoplasma • Sterility • Pyrogen and endotoxins • Adventitious viruses • Residual virus • Replication-competent vector	• ▲ (USP 1-May-2020) • Sterility • Pyrogen and endotoxins • Adventitious viruses • RCV	• Mycoplasma • Sterility • Pyrogen and endotoxins

Sampling Issues

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See *Stability-Protocol Development* for additional considerations. Samples from each lot should be retained in case of a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling scheme is highlighted in safety testing for adventitious agents or in assessment of RCV for gene-modified cell or viral gene therapy products. In such cases, process validation assists in determining the appropriate statistically based sampling design.

Safety

Safety testing for gene therapy products focuses on three issues: 1) detecting contamination from adventitious sources during product processing, 2) preventing the use of packaging cell lines and plasmids that potentially permit genetic recombination between vectors and the packaging cell lines or plasmids—or among the vectors themselves, and 3) testing the final product to ensure a safe level of undesired genetic and/or structural variants or other viruses used in processing.

The primary means of assessing safety are the performance of biological assays to measure adventitious agents directly. Molecular biology-based assays that measure adventitious agent DNA or RNA or detect undesired genetic variants are also used. Although live genetically engineered vectors officially fall outside its scope, the detailed information available in the ICH Q5A guideline, presented in ⟨1050⟩, should be consulted because the principles apply.

VIRAL GENE THERAPY PRODUCTS

One of the primary safety concerns associated with viral vectors used for gene therapy is the occurrence of undesired genetic variants. Among them the most critical type, and probably the best studied, is RCV. RCV is more clearly defined for

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replication-incompetent viral vectors, but for conditionally replication-competent viruses it refers to undesired genetic variants that have lost selectivity toward the target cells and thus might raise safety concerns. Regardless of the virus, these concerns are based on the potential lack of predictability for the pathogenicity of a contaminating virus for a specific route of administration, particularly if it is not the normal route of infection or if humans are not a natural host for the virus. The pathogenesis of a wild-type adenovirus infection is known but may not be predictive for the routes of administration employed with recombinant adenoviral vectors. For replication-incompetent adenoviral vectors, a limit of 1 RCA per 3×10^{10} viral particles is currently considered acceptable (see the FDA's *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*].

Typically, RCA levels are determined by a cell-based assay that allows amplification of the RCA while preventing replication of the product. The cell line most often used for amplification and detection of RCA is the A549 cell line. However, some recombinant adenoviral vectors express therapeutic genes that interfere with analysis on A549 cells. In such cases, ▲another cell line is used. The▲ (USP 1-May-2020) cell line is chosen on the basis of resistance to the effects of expression of the transgene and with subsequent passage of cell lysate or supernatant onto A549 cells for amplification and detection of the RCA. RCA is most often detected by visual observation of the cytopathic effect, but it can also be detected in the A549 cell culture by immuno- or polymerase chain reaction (PCR)-based methods.

Quantitation of the RCA level is based on the quantity of sample tested and the detection limit of the assay. Typically, RCA bioassays are validated as being able to detect 1 plaque-forming unit or infectious unit of RCA in the test sample over a wide range of test-sample sizes. Test-sample sizes can range, but they are typically based on the FDA RCA acceptance limit. To verify detection limits, include spike controls as part of the test, even with validated assays. For recombinant adenoviruses produced using HEK293 cells, RCA detection by PCR on the final products or the progeny virus amplified in HEK293 cells can be confounded by detection of residual HEK293 host-cell DNA (detection of the E1 region). PCR assays, however, can be designed to specifically quantitate host cell DNA contamination and can be made specific to particular forms of slow-growing RCA. Quantitative PCR assays can be used in conjunction with a cell-based method for precise quantitation of RCA levels. When a tested sample is found to be positive, the identity of the RCA is usually confirmed by conducting PCR analysis. This rules out the possibility that contamination of the assay by exogenous wild-type adenovirus or other adventitious agents is responsible for the positive result.

For conditionally replication-competent adenoviruses or other replication-competent viral vectors, testing for RCV or undesired genetic variants is usually more complicated and vector specific. Usually one or two nonpermissive cell lines that are not target cells are infected with the replication-competent virus in attempts to produce progeny virus. In order to generate a sufficient quantity of progeny population for analysis, analysts subject the infection to multiple passages and extended culture time. Two normal fibroblast cell lines that are easy to culture, WI-38 and MRC-5, have been used as the model nonpermissive cell lines for detecting RCA in replication-competent adenovirus products. Even after multiple passages on the nonpermissive cell lines, it may be necessary to amplify the progeny (which tend to appear only in minute quantities) in permissive or packaging cell lines to a sufficient quantity for subsequent testing. The resulting progeny should be tested for changes in biological selectivity and genetic composition. Usually the genetic characterization of the progeny population includes restriction enzyme mapping followed by Southern blotting, PCR, or nucleotide sequencing. After the genetic elements unique to RCV or undesired genetic variants are identified, quantitative PCR assays can be designed to monitor the level of RCV after amplification in nonpermissive cell lines or sometimes, if the sensitivity is adequate, directly in the final product without biological amplification. Using a spike control in the biological assay for detecting RCV is encouraged but may not be applicable to all cases. Currently there is no specified acceptable limit of RCA for conditionally replication-competent adenovirus, although clinical safety has been reported for an oncology application with several thousands of RCA per dose.

For retroviral vectors, testing for RCR is required for cell banks, viral vector production lots, and any resulting ex vivo product lots (see FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*). Standard assays have been designed to detect replication-competent MLV. The pathogenesis and potential long-term toxicity of low-level amphotropic MLV in human beings is not known. Methods commonly used to detect RCR include an amplification of virus titer by application of product to a replication-permissive cell line such as *Mus dunni*. Because infection is limited by the ability of a virus to reach the cells by means of Brownian motion, procedures (e.g., centrifugation and filtration) that physically bring the virus into contact with the cells can be used to enhance detection. However, high-titer recombinant vector can interfere with the detection of low-level RCR, and this interference may be enhanced by such methods. Infected cells are passaged several times to allow viral replication. Culture medium is harvested at the end of the culture period, and RCR is detected by using an indicator cell line. If the product is an amphotropic MLV, RCR can be detected by using a feline cell-based PG4 S+L– assay, a mink cell-based MiCl S+L– assay, or a marker rescue assay. In S+L– assays, the RCR expresses proteins that lead to transformation and subsequent plaque formation on the monolayer. In a marker rescue assay, RCR infects a cell line that expresses a retroviral vector encoding a marker gene such as β-galactosidase, drug resistance, or a fluorescent protein. The vector is packaged by the proteins supplied to it in trans by the RCR. The potentially vector-laden supernatant is transferred to naive target cells that are then screened for expression of the marker vector.

Testing for RCR is performed by co-cultivation of the cell line or amplification of vector supernatant with an RCR replication-permissive cell line, typically *M. dunni*, for several passages. Culture medium is harvested at the end of this co-cultivation process and applied to an appropriate indicator cell line as described above. Note that artifacts may be generated during the co-cultivation assay by expression of an endogenous virus in the permissive cell line or by fusion if the vector-producing cell line is cultured directly with a marker rescue cell line. In addition, co-cultivation may not be possible for ex vivo cell products that have specific culture requirements or limited culture life spans.

Methodologies for testing the presence of RCR in crude, purified bulk or final vector products are not specified. The FDA has deposited a reference standard of an amphotropic hybrid MLV with the American Type Culture Collection (ATCC). This viral stock has been assigned a label titer and should be used in assay validation. Method validation should demonstrate the ability to reproducibly detect a single RCR particle in individual product types because the product and its related impurities can interfere with the detection of RCR. Currently, there are no acceptable limits for RCR contamination in products. Any product lot found to contain RCR cannot be used for human use. Reference standards for assessing RCV in other viral vectors including

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ecotropic, xenotropic, or pseudotyped MLV, adenovirus, and lentivirus have not been developed. The adenovirus reference material, which consists of wild-type human adenovirus type 5, has been used as a spike control and during validation of RCA assays, but this practice may not be applicable to all RCA assays. Amplification and detection of replication-competent human immunodeficiency virus (HIV), especially its pseudotyped variants, may warrant special containment and handling procedures.

Additional safety testing usually focuses on methods similar to those described in *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Safety Tests—Biologicals* and ⟨71⟩. For viral gene therapy vectors produced using a human cell line, performance of the in vitro adventitious agent bioassays using three cell lines is recommended. For adenoviral vectors, specific tests for adeno-associated virus are also recommended. For adeno-associated virus, specific tests for adenovirus and herpes virus are recommended. Material for testing should be derived from the stage of manufacture that provides the greatest chance of detection, which could be prebulk (e.g., late-stage fermentation), the bulk, or the final product.

NONVIRAL GENE THERAPY PRODUCTS

Safety testing usually focuses on methods similar to those described in ⟨88⟩ and ⟨71⟩. Safety testing should be performed on nonviral formulated material. If the shelf life of the formulated nonviral product is very short, then the components should be tested individually.

Safety testing for undesired genetic variants that might emerge during the manufacturing process in nonviral gene therapy products is similar to that for RCV testing for viral vectors but with more vector-specific considerations. Typically, molecular biology-based methods are applied to the final product to test for variants. When genetic stability is established by process validation, the assays for monitoring the levels of undesired genetic variants may be limited to restriction enzyme mapping followed by further confirmation of critical genetic elements (such as transgenes or regulatory elements) by PCR or Southern blotting.

Dose-Defining Assays

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision. An assay that measures therapeutic activity of the product is referred to as a potency assay, and it is designed to measure product function. The design of the assay depends on the type of product. In the case of chemical and protein drugs, the assays measuring the amount of active ingredient (dose) are referred to as strength assays. Product dose can be defined as the concentration or amount of the drug product administered to the patient, and it is typically measured as product mass.

Particle concentration is a commonly used measure for viral vector product dose. Particle concentration may be measured by physical, biophysical, or in vitro cell-based assays. For example, quantitation of purified adenoviral particles may be determined by using the optical density of a solution of virus in 0.1% (w/v) sodium dodecyl sulfate (SDS) solution at 260 nm, because a relationship between absorption and particle concentration has been published for adenovirus. The particle number concentration is equivalent to the product of the absorbance at 260 nm in a 1-cm cell, the dilution factor, and 1.1×10^{12} particles. A method that has become standard in determining particle concentration is integration of viral peak area of 260 nm and/or 280 nm absorbance against an authenticated reference standard in an anion-exchange resin-based HPLC assay. Compared to the optical density method, the HPLC method has the advantage of eliminating the interference of free DNA and/or capsid proteins on quantitation of viral particles. An adenoviral reference material (ARM) from ATCC has an HPLC-determined particle concentration established from a large-scale collaboration that involved many laboratories. Whenever possible, the ARM should be used to calibrate the internal HPLC method and reference material.

Virus concentration can also be assessed by the measurement of selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus must be lysed, and the structural proteins must be separated by using an appropriate, high-recovery chromatographic procedure (e.g., reversed-phase HPLC). The chromatographic separation and the identity and the purity of the selected structural protein must be verified during assay validation by methods such as SDS polyacrylamide gel electrophoresis (SDS-PAGE), peptide sequencing, and mass spectroscopy. The selected structural proteins can be quantified, for example, by integrating chromatographic peaks at 214 nm and comparing the area to that of an authenticated reference standard. The virus concentration can then be calculated based on the molecular mass, the copy number, and the measured mass of the protein. Very importantly, the virus concentration can be estimated simultaneously for multiple structural proteins, which allows the use of this assay in relatively impure virus preparations. This method has been applied to adenovirus and should be applicable to other viral vector types.

Biophysical methods of determining particle number include direct quantitation of vector nucleic acid by radiolabeled-probe hybridization and indirect quantitation by amplification of template nucleic acid [e.g., PCR and reverse transcription (RT)-PCR] or by signal amplification (e.g., branched-chain DNA using multiple-probe hybridization).

In cases where biophysical methods are not available, bioassays that measure gene-vector titer have been used. These involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some measure of the product uptake. Methods for quantitation or estimation of the number of infection, transfection, or transduction events include plaque-forming unit assays, tissue culture infectious dose assays based on cytopathic effect of 50% tissue culture infectious dose ($TCID_{50}$) or immunofluorescent detection of an expressed vector protein, or a quantitative DNA-hybridization assay. Examples follow: For replication-competent adenoviral gene therapy products, the ARM available from ATCC has a defined range of $TCID_{50}$ titer determined via a collaborative effort. Whenever applicable, it should be used in validation of an internal reference standard or assay control of infectious titer assays. However, because of the likelihood of genetic differences between the ARM, which is wild-type human adenovirus type 5, and the replication-competent adenoviral gene therapy product, it may not be reasonable to normalize the titer of the vector of interest to that of the ARM.

For retroviral or lentiviral gene therapy products or AAVs that carry a selectable marker (e.g., that for neomycin resistance) or a reporter gene (e.g., β-galactosidase) in addition to the therapeutic gene, the infectious titer is commonly determined by measuring the number of transduced or infected cells expressing these nontherapeutic proteins. Vector titer is typically reported as the number of colony-forming units (cfu) per mL for cells transduced with viral vectors that contain drug-resistance markers

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and are selected for growth in drug-containing medium. Titer based on β-galactosidase can be expressed in terms of blue cfu/mL after staining and counting the cells that convert the β-galactosidase substrate X-Gal into a blue chromophore. For vectors without a marker gene, quantitation of transduction has been measured precisely by using quantitative PCR or has been estimated by hybridization methods.

Most nonviral gene therapy products contain plasmid DNA, and their usual measure of dose is the DNA mass. The DNA mass may be determined in the formulated state, and, if recombinant protein is included in the formulation, the total combined mass of all formulation components based on a specific ratio can be used. DNA concentrations greater than 500 ng/mL are most simply determined by using optical density measurement at 260 nm. This method is not generally applicable to lipid-formulated DNA. Because RNA and proteins also have significant absorbance at 260 nm, other analyses must be performed to demonstrate minimal contamination with RNA, protein, or residual host-cell chromosomal DNA. Dyes that specifically bind to double-stranded DNA allow accurate measurement of DNA concentrations of less than 500 ng/mL when calculated against an authenticated DNA standard curve. PicoGreen is one such fluorescent dye, and it is minimally affected by single-stranded DNA, RNA, proteins, salts, and detergents. The fluorescent dye Hoechst 33258 also binds to both double-stranded and single-stranded DNA and it can be used to determine DNA concentrations as low as 0.3 ng/mL. The Hoechst 33258 does not bind to protein or RNA, and it can accurately determine the DNA concentrations in crude samples.

Methods such as capillary electrophoresis and HPLC employing an authenticated reference material can also be used to determine the strength of nonviral products.

Potency

Potency is defined as the therapeutic activity of the drug product. Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*). Potency can be assessed by in vitro or in vivo bioassays. It is not uncommon for these assays to have coefficients of variation between 30% and 50%, although stringent assay design with good statistical consideration could help reduce assay variation. These assays require a well-defined, representative reference material that can be used as a positive control for the assay and/or in calculation of the relative potency of the test article. The general consideration for bioassays in current *USP* chapters on design and development of biological assays should be applied to the potency assay design for gene therapy products. The positive control qualifies the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. High-precision assays are more effective tools in monitoring product quality. Information about potency assay variability should be incorporated into the stability study design and the proposed statistical approach to assignment of expiration date (see *Stability*).

Bioassays employed to measure the potency of viral and nonviral gene therapy products generally involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some functional measure of the expressed gene of interest. Functional assays for the therapeutic gene (e.g., those measuring enzyme activity and cell growth stimulation or inhibition) should generally be used instead of analytical methods such as enzyme-linked immunosorbent assay (ELISA). When the biological function of the expressed transgene exhibits a broad range of activities or only generates semiquantitative results, the ELISA or other immunological or biochemical readouts can be used as a surrogate potency assay with a tight specification range if extensive characterization data is available to demonstrate that all expressed protein is biologically active. For example, in the case of a gene therapy product expressing a cytokine, cytokine expression is usually quantified by ELISA first, and the result is used to adjust the sample dilution for the functional assay. The potency of such vectors may be better controlled by the ELISA quantitation results, but the biological activity of expressed cytokine could be used to verify that the measured mass is biologically active without the requirement to meet a narrow specification range for the biological activity itself.

HPLC or flow cytometry, which provide information about the level of expression but only infer function, have also been used in a context like that described for immunoassays. In addition, for viral vectors, infectious titer measurements by themselves are generally not considered an adequate measure of product potency. For example, the $TCID_{50}$ titer or plaque-forming-unit assays for adenoviral vectors on HEK293 cells can indicate that the infectivity of adenovirus is preserved but do not confirm that the adenoviral product has maintained full biological function(s), especially transgene biological activity. The design and ultimate suitability of an assay system for determining product potency depends on the relationship between the intended human target cell in vivo and the following: 1) the transduction or transfection efficiency of the cell line used in vitro; 2) the protein expression levels; and, 3) the duration of expression required for the therapeutic effect.

In vivo tests can also be used to measure vector-product potency. Readouts can be based on a response per animal (e.g., blood levels of therapeutic protein 24 h after treatment) or a group response rate (e.g., percentage of animals that elicited an immune response or survived virus challenge). The availability of an appropriate in vivo test system depends on the vector-host range (for viral vectors), the pharmacokinetics and biodistribution of the vector and the resulting gene product relative to its human counterpart, and the time frame required to observe the therapeutic effect or surrogate. Issues of cost, facilities, validation, and ethics determine the practicality of an in vivo potency test.

Purity

Analytical methods that separate, isolate, and specifically quantify the intended active product components determine product purity. Impurities are either product- or process-related components that can be carried through to the final product. The manufacturing and purification process should be optimized to consistently remove impurities while retaining product activity. The requirement to test for a particular impurity for product lot release depends on the following: 1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity through process validation, and 2) the potential toxicity associated with the impurity.

Examples of process-related impurities associated with gene therapy products include residual medium components for the production [e.g., fetal bovine serum (FBS), antibiotics, cytokines, and *E. coli* chromosomal DNA in a plasmid product], ancillary products used in downstream processing (e.g., nucleases such as DNase I), and residual moisture for lyophilized vector products. Impurities may be bioactive (e.g., cytokines and hormones) or immunogenic (e.g., product aggregates, degradation products,

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plasmid-selection markers, and nonhuman-derived proteins), or they may have other deleterious effects (e.g., they may compete with the product) if administered at a dose equal to that of the product. Product-related impurities are specific to each product type. Examples include nicked plasmid forms in nonviral products and defective or immature virus particles in retroviral or adenoviral vector products. Analytical methodologies to assess purity require quantitation or physical separation of the intended product from its impurities. Common sense should drive the need to quantify specific impurities. Suitable validation of the manufacturing process may limit the need for specific lot-release testing for impurities. Manufacturers may place an emphasis on demonstrating the consistency of the product–impurity profile.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

VIRAL GENE THERAPY PRODUCTS

Product-related impurities for viral vectors include aggregates and defective and immature particles that may be produced during the manufacture or purification of the recombinant vector. Aggregates of vector may form if the product is highly concentrated, stored under certain conditions (e.g., under a certain pH or temperature), or reconstituted after lyophilization. Assays to detect aggregates include particle size analysis by laser light-scattering and the use of nonreducing, nondenaturing PAGE, followed by staining of the gel or transfer and detection of viral proteins by Western blot analysis. Sedimentation rate analysis also allows separation of aggregates from monomers based on size. Optical density analyses of light scattering are also used to assess vector aggregation.

Defective particles are viral particles that do not contain the appropriate recombinant genome—that is, they contain some other nucleic acid or contain no genome at all, or the vector has some missing, defective, or otherwise altered structural component that impairs its ability to transduce a cell. For viral vector systems that have capsomeric symmetry that requires the appropriate nucleic acid incorporation for configuration, empty particles may be readily distinguished from those carrying genomes. For enveloped viruses, empty particles may not be as readily separated from those with encapsidated nucleic acid.

For some viral vector products, active viral particles can be separated from defective particles by using analytical HPLC. Anion-exchange resins have been used to separate active adenovirus from defective virus particles. However, this method might not be useful for an adenoviral vector purified by anion-exchange chromatography unless the resin for the assay is different from that used during manufacture. Depending on the nature of the viral vector and its nonactive or defective forms, other methods of separation, such as equilibrium centrifugation in a cesium chloride density gradient, may need to precede the quantitation of the active particle. Ideally, the method of separation will allow quantitation.

Defective particles that carry a non-cell-derived oncogene or other undesirable genes may pose a special concern. For example, in murine-based retroviral packaging cell lines, small viral elements called VL30 sequences can be packaged in about one-third of all particles. Assays may be needed to quantify specific defective particles if they are known to be present in quantities sufficient to pose a safety concern.

Virus quality and the comparability of preparations can also be assessed by measuring selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus is lysed, and the structural proteins are separated by using reverse-phase HPLC or some other high-recovery chromatographic procedure. The chromatographic separation should be validated, and the identity of the selected structural proteins should be verified by methods such as SDS-PAGE, peptide sequencing, or mass spectroscopy. Fingerprinting of the batch can be conducted based on quantification of the selected structural proteins and comparison to a reference standard. When the method incorporates mass spectroscopy, impurities such as structural variants can also be identified. For adenovirus preparations, some precursor and most mature virion proteins can be detected and distinguished, thus allowing monitoring of the product and of the immature virion forms.

Host cell-derived proteins may be considered impurities for some viral vector products and may be separated and quantified by PAGE or HPLC or detected by amino acid analysis, Western blot, or immunoassay-based methods. However, for enveloped viruses such as retroviruses, host cell-derived membrane proteins are an integral part of the product. In those vector systems, it may be difficult to determine the presence of contaminating exogenous host-derived proteins.

Presence of specific process-related impurities depends on the manufacture and purification process of each vector or product type. However, most products need to be tested for residual endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩). Acceptable limits of endotoxins have been determined and can be directly applied to viral vector products. Although genomic DNA derived from continuous cell substrates used to manufacture biological products historically has been considered potentially tumorigenic, recent studies suggest that the risks are very low. However, every attempt should be made during process development to reduce levels of contaminating DNA. The need to test for residual DNA as part of product lot release should be evaluated on a case-by-case basis and may depend on the size distribution of the DNA, its association with the product or its formulation components, and the product's route of administration. Quantitative PCR assays can analyze the amount of residual host-cell DNA by using primers designed to amplify evolutionarily conserved and abundant target sequences such as 18S for HEK293 cells.

Quantitation of residual serum components such as bovine serum albumin (BSA) can be achieved by using ELISA and a BSA reference standard. Researchers may need to develop specific functional or immunological methods for other ancillary products, including other culture media or purification process components such as cytokines or enzymes (e.g., deoxyribonuclease 1 or benzon nuclease).

NONVIRAL GENE THERAPY PRODUCTS

A plasmid used as a drug substance is considered a well-characterized biologic, and key impurities from the manufacturing process are well known. Testing is usually performed on each individual component: the plasmid DNA, lipid or lipoplex reagents, and protein components if any are present in the formulation. Plasmid DNA is characterized for a variety of impurities, including residual host-cell DNA, residual RNA, and residual protein. Residual protein testing is frequently included in lot-release testing.

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Optical density ratios (usually the measurement at 260 nm ▲is compared to the measurement▲ (USP 1-May-2020) at 280 nm) are frequently used in purity specifications for plasmid DNA.

In addition, the plasmid DNA should also be characterized with regard to its conformation in solution. Plasmid DNA exists as monomeric supercoiled, relaxed monomer, and linear forms. Because all forms can be generated during large-scale fermentation, and data about their relative in vivo potency is scarce, the relative quantity of each form is monitored to verify batch-to-batch consistency in the relative amounts of each conformation. Agarose gel electrophoresis can resolve each of these forms but is not highly quantitative for each individual species. Analytical anion-exchange HPLC serves as a quantitative assay for monomeric supercoil and other forms, including concatamers. Other analytical methods that have been valuable for characterization of plasmid constructs during process development and validation such as capillary gel electrophoresis (CGE), linear-flow dichroism, and atomic-force microscopy are also viable methods to assess the purity of a given plasmid preparation. The most appropriate method for lot release depends on how each plasmid conformation affects product potency. Specific details for each of these methods are outlined in *Nucleic Acid-Based Techniques—General* ⟨1125⟩.

Tests should be conducted for process-related impurities such as residual organic solvents (phenol, alcohol), salts, and certain antibiotics such as kanamycin used during the fermentation process. Lipid and lipoplex formulation components must also be tested for their chemical purity. Testing for specific chemical impurities is commonly performed by using GC–MS, HPLC, or TLC methods. If protein is part of the formulated complex, then the protein must also be tested for purity. HPLC is capable of detecting trace amounts of residual antibiotics and can therefore be used during process validation or lot-release testing to confirm that they have been effectively removed. The specifics of these methods are outlined in *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩ or in *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

Bacterial protein, DNA, RNA, and endotoxins are the major types of host-derived process contaminants. Standard protein assays (e.g., Lowry, Bradford, or Coomassie), PAGE followed by silver staining or Western blot analysis, or ELISA can be used to detect residual host protein in the nanogram range. Host chromosomal DNA can be detected by slot blot hybridization (detection in picogram range) or by real-time PCR (detection sensitivity < 1 pg) using highly conserved target sequences (e.g., 18S for *E. coli*). PCR assays for this purpose must use recombinant polymerases that are highly purified to minimize residual bacterial DNA for which the presence can create background signals. PAGE or agarose gel electrophoresis followed by fluorescent dye staining can be used to detect residual RNA. Quantitation may not be required because of the labile nature of RNA and the low-level toxicity associated with it. The *Limulus* amobocyte lysate (LAL) test is the most sensitive and widely used method for endotoxins determination. Colorimetric assays offer sensitivities of 0.005 EU/mL. Details of the methods described here are outlined in ⟨1057⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩, ⟨1125⟩, and ⟨85⟩.

LYOPHILIZED VIRAL AND NONVIRAL VECTOR PRODUCTS

Residual moisture can affect the stability of a lyophilized vector product. FDA's *Guideline for the Determination of Residual Moisture in Dry Biological Products* recommends a 1% residual moisture level, although data indicating no adverse effects on product stability at higher levels is considered acceptable. Residual moisture levels can be determined by using a standard method (see *Water Determination* ⟨921⟩) that is compatible with the formulated product.

Identity

Lot-release testing for gene therapy products must include an identity test. This test clearly identifies the product and confirms that inadvertent substitution with another product has not occurred. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous gene-modified cell therapy product at a facility where multiple patient products are manufactured than for a viral vector product produced at a site that manufactures a single vector product.

VIRAL GENE THERAPY PRODUCTS

For characterization purposes, restriction enzyme mapping and sequencing of the transcription unit DNA are the most commonly used approaches. PCR-based methods, restriction enzyme mapping, and transgene-expression-based immunoassays are commonly used to confirm the identity during lot-release testing.

NONVIRAL GENE THERAPY PRODUCTS

Restriction enzyme mapping is the most common identity method for plasmid-based products. The number of enzymes used to create the vector fingerprint will vary according to the complexity of the DNA and the degree of similarity between multiple products. If lipids, lipoplex agents, or proteins are used to formulate the DNA, then their identity must also be tested and confirmed. Lipids and lipoplex components may be identified by procedures used for traditional pharmaceuticals, such as GC–MS and TLC. Protein components of the formulation may be identified by peptide mapping or other means outlined under ⟨1057⟩.

STABILITY

The shelf lives of gene therapy products vary widely depending on the nature of the product, its intended clinical use, its specific attributes, and the recommended storage, packaging, and shipping conditions. Therefore, it is difficult to draft uniform guidelines regarding stability-study duration and testing frequency for all products. In all cases, the study should be designed on the basis of scientifically sound principles and approaches and a comprehensive understanding of the final therapeutic product and its intended use. The product's stability during in-process hold steps, cell and virus banks, critical raw materials,

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and reference standards also must be assessed. A well-designed and executed stability program will provide a high degree of assurance that the product is stable within the specified shelf life.

For viral and nonviral vector gene therapy products and gene-modified cellular gene therapy products that are not patient-specific, the selection of batches to support license application and final-product labeling should be carried out in accordance with the principles of stability testing, such as those described in ICH guideline Q5C and ⟨1049⟩. Stability data should also be collected for bulk material and at other in-process points if material is stored before final processing and filling. Issues related to the stability of cell-based products are addressed in ⟨1046⟩.

Nonviral DNA plasmid vectors are often formulated with specific mixtures of lipids, proteins, or lipoconjugates to form liposomes or encapsulated complexes. Depending on the formulation, a shelf life of hours to years can be attained. Where a product has a short shelf life, the final formulation may require preparation at the clinic just before administration. Instability is frequently observed as aggregation and precipitation. Formation and stability of the formulated complex should be characterized and established by validation studies during product development. Stability data should also be collected for major components of the formulated complex, such as the lipids, the liposomes, and the DNA itself.

Stability-Protocol Development

Stability studies verify that the storage conditions maintain the purity and potency of the product for a defined period so that product administered to the patient is still capable of meeting the stability specifications. These specifications may differ from the manufacturing release specifications, but they must be verified with clinical data. Formal stability studies to support licensure as well as plans for gathering early-phase product stability information should be detailed in a written plan that describes how data will be collected and analyzed to support the product's expiration period. Protocols should follow the format recommended in existing guidelines and should include the scope, storage conditions, number of lots that will be tested, test schedule, assays that will be used, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the problems the product may encounter during manufacturing, shipping, and processing at the clinical site (see *Accelerated and Most Appropriate Challenge Conditions* below). The study design should also incorporate the latest knowledge in the biological sciences and should address existing regulatory requirements. For instance, if the product's final formulation is performed at the clinical site, stability studies on this final formulation should be performed to establish the time and conditions under which the product can be held.

Stability assessment should include an evaluation of product functionality (potency). The potency assay often has a high degree of inherent variability. Measuring and calculating the decay of product activity by employing the standard statistical methodologies may require multiple, frequent sampling intervals over an extended period of time and may require analysis of more than three production lots to compensate for assay variability. Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Initial studies are also useful for determining which assays are stability indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of gene therapy products, the development of assays that would address these unique characteristics is encouraged.

Accelerated and Most Appropriate Challenge Conditions

The stability-indicating profile of a gene therapy product varies over time under the influence of a wide variety of environmental conditions, including temperature, extremes in physiological storage conditions, and light. Multifactorial degradation pathways must be considered when researchers investigate the effects of these parameters on the stability of the product. Studies should include conditions that are outside the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation due to shipping to hot or cold climates, hypobaric conditions experienced in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite. A short exposure to an environmental condition well outside of an established limit and a long exposure to an environmental condition just outside of an established acceptable range may be equally detrimental to the overall stability profile. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Give special attention to products stored in fluids that contain light-sensitive or reactive components that may give rise to cytotoxic by-products.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to determine how the product degrades and which assays are stability indicating. These studies can be the same as some of those mentioned in the preceding paragraph. Other studies include placing a product at 37°, or at 18° when its normal storage temperature is 25 ± 2°, or placing a lyophilized product in a high-humidity environment. Such studies should be performed before formal stability studies begin so that the latter can incorporate the validated stability-indicating assays.

STORAGE AND SHIPPING

Appropriate conditions are chosen to preserve the purity and potency of the product so that its specification and those of its ingredients are maintained throughout storage, shipping, and handling at the clinic. Initial studies must be conducted before patient administration to determine acceptable storage, shipping, and handling conditions. The initial storage and shipping conditions need not be those envisioned for the commercial product but should ensure that the product specifications are maintained beyond the initial expiration date. For products with short shelf lives, storage and shipping conditions, even within a medical center, must be considered at the same time because shipping constitutes the bulk of storage time after manufacturing. Give special consideration to the ability of gas to permeate the shipping container, especially if the gene therapy product is

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stored or shipped on dry ice. Once stability-indicating methods are developed and the final storage and shipping conditions are chosen, these must be validated as discussed under *Stability*.

Most products with limited shelf lives are shipped by reliable overnight courier systems. In some cases, highly fragile products are hand-carried onto commercial aircraft. Special permission must be obtained by commercial carriers if scanning by airport X-ray equipment must be avoided. Cargo shipping studies should be designed during the development of packaging systems to identify stresses to which the product may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will tolerate, and protect the product against, the extreme conditions of shipping.

Most gene therapy products can be either lyophilized or formulated by means similar to those employed for many recombinant proteins or cell therapy products. These storage formulations typically have expiration periods longer than 1 year and no unusual shipping requirements. Nonviral gene therapy products, which may be unstable in their final formulation, can have similar expiration periods if they are stored in a multiple-vial kit with the nucleic acid in 1 vial and a carrier, such as lipids, in the other. The final formulation is performed at the medical center just before administration.

LABELING

Product labeling is regulated by the FDA, and compliance with existing policies is required. Because gene therapy products are regulated biologics, their labeling is subject to these rules. Biologics and devices must meet labeling requirements specific to the container and the package (21 CFR 610 and 801, respectively). Both the container label and the package label must include the expiration date. If the container is packaged, then the recommended storage conditions should be included on the outer package label. If the container is not packaged, the recommended storage conditions and all other requirements of a package label must appear on the container. Labeling must also comply with relevant national and international requirements.

If a product must be applied to the patient in a particular physical orientation or in a specifically defined area, labeling that indicates the correct orientation and/or area should be apparent even after the package is opened. Unless the product has been screened for pathogenic or microbial contaminants before release, appropriate biohazard labeling may be required. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life. Clinical procedures must be scheduled around these crucial time frames. For patient-specific products, the patient's full name, initials, or a combination of these will need to appear on the labeling, in addition to lot designation, to ensure that the product is administered to the appropriate patient.

REGULATIONS AND STANDARDS

The technologies involved in manufacturing gene therapy products have been widely documented in the literature and continue to evolve. These products can be regulated as drugs or biologics, or uncommonly as devices, depending on how they are manufactured and used. The novel approaches permitted by these technologies may make it difficult to determine which FDA centers will be involved in their regulation, and the FDA has advised manufacturers to seek clarification in the early stages of development. Currently, the Center for Biologics Evaluation and Research (CBER) regulates most human gene therapy products. CBER relies on both the Public Health Service Act and the Federal Food, Drug, and Cosmetic Act. Regulation is the same as that for biotechnology-derived products. The general requirements are described primarily in 21 CFR. The federal government has issued many guidance documents as *Points to Consider* or *Guidelines* (see www.fda.gov). ICH guidance documents for many of the quality-related areas are relevant in varying degrees to qualifying gene therapy products (although some products are nominally outside the scope of the guidance documents, the principles still apply; see www.ifpma.org or www.ich.org). Some of these documents are reproduced in *USP–NF* as general chapters. ICH has also held a number of meetings about gene therapy products and has a Gene Therapy Discussion Group (GTDG) that addresses current issues in gene therapy product development and research and has released several ICH Considerations that reflect harmonized principles. The National Institutes of Health (NIH) has published *Guidelines for Research Involving Recombinant DNA Molecules* that require NIH review of research, including clinical research or trials conducted or sponsored by institutions that receive NIH funding. These guidelines apply to many gene therapy products.

Biological and biochemical standards for quality assurance (QA) of the production and analysis of gene therapy products are highly desirable. The diversity of gene therapy products, in particular viral vectors, has so far limited the development of standards that have wide applicability. A MuLV RCR preparation (VR-1450) with an assigned infectivity titer is available from ATCC for testing murine retroviral vectors for the presence of RCR. A wild-type adenovirus type 5 reference standard with assigned particle number and infectivity titer for characterization of adenoviral vectors is also available from ATCC. A working group has been set up to oversee the development of an AAV reference standard. However, several obstacles to choosing, developing, establishing, and circulating suitable standards are apparent. These include decisions about which virus serotype will be most commonly and successfully used for gene therapy, availability of GMP prepared materials, safety, long-term stability, transportation, and initiation and completion of collaborative studies to evaluate candidate standards. Thus, development of standards for other viral vectors, including lentiviral-, herpes viral-, and poxviral-vectors, remains challenging.

New methodologies, including proteomics, novel nucleic acid technologies (NATs), protein modification methods, and stem cell isolation and culture, are now available and, in many cases, are applicable to the development, characterization, and analysis of gene therapy products. In addition, the use of synthetic polymers both for the modification of existing viral vectors and for the development of chemically dynamic synthetic vectors provides advantages, e.g., improved systemic circulation, better targeting and delivery, and lower levels of immunostimulation and inflammation. The availability of defined stem cell populations and improved engrafting methods should lead to greater effectiveness of ex vivo transduced cells used in gene therapy protocols. The introduction of new methodologies will require the continual review and regulatory oversight to ensure the quality and safety of gene therapy products of the future.

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GLOSSARY

Adenovirus: Virus belonging to the family Adenoviridae of DNA viruses having a nonenveloped virion with 252 capsomeres and a diameter between 70 and 90 nm; a single linear molecule of double-stranded DNA (36–38 kb); at least 10 structural ether-resistant and acid-stable proteins; virions are released by cell destruction.

Adenovirus-associated virus (AAV)**:** Human parvovirus contains a single-stranded DNA genome and depends on helper viruses (adenovirus, herpes virus, or vaccinia virus) for replication. Without co-infection, the wild-type virions integrate at a specific site on chromosome 19 and remain latent.

Adventitious agent: A foreign agent that is introduced accidentally or inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

Amphotropic virus: A virus that infects and replicates in cells from multiple species.

Ancillary materials: Components used during manufacturing that are not intended to be present in the final product. Examples: growth factors, monoclonal antibodies, cell-separation devices, and media components.

Autologous: From one's own body.

Base pair: Two nucleotide bases on different strands of the nucleic acid molecule that bond together.

Bioassay: Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*▲in this *Glossary.*)▲ (USP 1-May-2020)

Biological product: Any virus, therapeutic serum, toxin, antitoxin, or "analogous product" applicable to the prevention, treatment, or cure of diseases or injuries in humans. (In this US FDA-derived definition, the term "analogous product" has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

Biotechnology: Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

Cell lines: Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or may be immortalized (modified to be able to replicate indefinitely).

Cell therapy: Therapy that uses whole cells to treat a disease, condition, or injury. Distinct from tissue and organ transplantation.

CFTR: Cystic fibrosis transmembrane conductance regulator.

cGMP: Current good manufacturing practices. The FDA outlined cGMPs in the 21 CFR, in the *Federal Register*, and its *Points to Consider.*

Complementary DNA (cDNA)**:** DNA synthesized from an mRNA rather than a DNA template. It is used for cloning or as a DNA probe for locating specific genes.

Cytokine: Any factor that acts on cells; usually a protein that promotes growth.

Cytoplasm: Cellular material that is within the cell membrane and surrounds the nucleus.

Cytotoxic: Able to cause cell death.

Differentiation: A process of biochemical and structural changes by which cells become specialized in form and function.

Ecotropic virus: A virus that infects and replicates in cells from only the original host species.

Electroporation: Method for enabling transfer of material into cells that involves use of a brief electrical field to create temporary pores in the cell membrane.

Enzyme-linked immunosorbent assay (ELISA): An immunoassay that utilizes an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

Enveloped viruses: Viruses containing a lipoprotein bilayer surrounding the capsid and acquired by budding through the cell membrane of the host cells.

Episomal: Pertaining to any accessory extra-chromosomal genetic material.

Ex vivo: Procedure performed outside of the living organism.

Fluorescence-activated cell sorter (FACS)**:** A machine that sorts cells based on fluorescent markers attached to them.

Formulated: Prepared in accordance with a prescribed method or conditions.

Fusion: Joining of the membrane of two cells, creating a daughter that contains some of the same properties from each parent cell. It is used in making hybridoma cells in which antibody-producing cells are fused to mouse myeloma cells.

Gene construct: Expression vector that contains the coding sequence of the protein and the necessary elements for its expression.

Gene therapy: Therapy that uses nucleic acid that is subsequently expressed as RNA or protein to treat a disease or condition. The US FDA defines gene therapy products as products containing genetic material administered to modify or manipulate the expression of genetic material to alter the biological properties of living cells.

Genome: Total hereditary material of a cell.

Germ cell: Reproductive cell (sperm or egg), gamete, or sex cell.

Graft-versus-host disease (GVHD)**:** Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

Growth factors: Factors responsible for regulatory cell proliferation, function, and differentiation.

Helper virus: Aids the development of a defective virus by supplying or restoring the activity of a viral gene or by enabling the defective virus to form a functional envelope.

Hematopoietic: Pertaining to or affecting the formation of blood cells.

Hepatocyte: The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells generally do not divide, but when injured they can divide and regenerate until the injured cells are replaced.

Herpes simplex virus (HSV)**:** A DNA virus that is a member of the family Herpesviridae. It can infect both warm- and cold-blooded vertebrates by contact between moist mucosal surfaces.

Human leukocyte antigen (HLA)**:** Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

Humoral: Pertaining to elements found in body fluids (for example, humoral immunity and neutralizing antibodies).

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Hybridization dot blot (DNA or RNA)**:** A technique for detecting, analyzing, and identifying protein; similar to the Western blot but without electrophoretic separation of proteins.

Immunoassay: Technique for identifying substances based on the use of antibodies.

Insertional mutagenesis: A type of mutation that is caused by the insertion of nucleic acid into a host-cell chromosome. There are multiple possible negative consequences of such an event, including death of a cell if an essential gene is inactivated or predisposition to cancer if a tumor suppressor gene is inactivated.

Integration: Assimilation (insertion via covalent binding) of genetic material (DNA) into the chromosome of a recipient cell.

Intrabodies: Intracellular antibodies that are not secreted and that are designed to bind and inactivate target molecules inside cells.

In vivo: Procedure performed in the living organism.

In vitro: Procedure performed outside of the living organism. It may involve cells or tissues derived from the organisms.

Leukemia: Malignant neoplasm of the blood-forming tissues.

Lipoplex: A formulation of lipids and polymers and/or proteins.

Liposome: A spherical lipid bilayer ▲or multiple lipid bilayers▲ (USP 1-May-2020) enclosing ▲one or more▲ (USP 1-May-2020) aqueous ▲compartment(s). See *Injections and Implanted Drug Products* ⟨1⟩.▲ (USP 1-May-2020)

Mock run: A test run that deliberately omits some critical reagents.

Monoclonal antibodies: Antibodies that are derived from a single cell clone.

Naked DNA: Isolated, purified, and uncomplexed DNA (no protein or lipid).

▲▲ (USP 1-May-2020)

Oncogenes: Genes associated with neoplastic proliferation (cancer) following a mutation or perturbation in their expression.

Oncogenic: Cancer-causing.

Packaging cell line: Cell line that produces proteins required for packaging and production of viral vectors in an active form but does not produce replication-competent virus. It complements at the protein level what the vector is lacking genetically.

Parvovirus: DNA viruses of the family Parvoviridae. Host range includes many vertebrate species. Small, linear chain, single-stranded DNA with terminal hairpin loops.

Plasmid: A small circular form of DNA that carries certain genes and is capable of replicating independently in a host cell.

Polymerase chain reaction (PCR)**:** Technique to amplify a target DNA or RNA sequence of nucleotides by repeated cycles of polymerase-based copying, resulting in geometric increases in copy number.

Potency: A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.

Process validation: Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.

Producer cell line: An established cell line used to produce virus vectors, often on a large scale.

Promoter: DNA sequence that is located at the front of a gene and controls gene expression. It is required for binding of RNA polymerase to initiate transcription.

Recombinant DNA: DNA produced by joining fragments of DNA from different sources by in vitro manipulations.

Replication-competent virus: A virus that can complete an entire replication cycle without a need for a helper virus; an autonomously replicating virus.

Restriction endonuclease: An endonuclease that recognizes a specific sequence of bases within double-stranded DNA.

Retrovirus: A virus that contains reverse transcriptase, which converts viral RNA into DNA that then integrates into the host cell in a form called a provirus.

Serum-free: Refers to cell growth medium that lacks a serum component.

▲**Southern blot:** Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane.▲ (USP 1-May-2020)

S phase: Synthesis phase. Part of the cell cycle during which DNA replication occurs.

Stem cell: Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.

Suspension culture: Cells capable of growth in suspension, not requiring substrate (attachment) on which to grow.

Transduction: Transfer and expression of genetic material into a cell by means of a virus or phage vector.

Transfection: Transfer of DNA into cells by physical means such as by calcium phosphate coprecipitation.

Transgene: Refers to the foreign genetic material delivered as part of a vector construct.

Vector: The agent (plasmid, virus, or liposome–protein or DNA–protein complex) used to introduce nucleic acid into a cell.

Viability: State of being alive and functional.

Virion: An elementary viral particle consisting of genetic material (nucleocapsid) and a protein covering.

Virus: Submicroscopic infectious agent that contains genetic information necessary for reproduction. It is an obligate intracellular parasite.

Western blot: An electroblotting method in which proteins are transferred from a gel to a thin, rigid support (e.g., nitrocellulose membrane) and detected by binding radioactively labeled antibody or antibody coupled to an enzyme, allowing use of a precipitating chromogenic or chemiluminescent substrate.

Xenogeneic: From a different species.

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APPENDIX

Lists of Relevant Regulatory References

Gene therapy products are regulated by the FDA as biologics, and therefore their manufacturing, testing, labeling, and other factors are subject to the requirements codified in CFR and FDA guidance documents (www.fda.gov). Additional guidance is provided in ICH guidelines (www.ich.org). Manufacturers of gene therapy products that seek markets outside the US should refer to regulatory documents from relevant countries. Beyond *USP* chapters, the following lists include regulatory documents, as well as best practices for the development, manufacturing, quality control, and quality assurance of gene therapy products.

▲Code of Federal Regulations (CFR)

Regulations	Title
21 CFR 210	Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General
21 CFR 211	Current Good Manufacturing Practice for Finished Pharmaceuticals
21 CFR 600s	Biological Products: General
21 CFR 610 Subpart G	General Biological Products Standards—Labeling Standards
21 CFR 801	Labeling
21 CFR 820	Quality System Regulation▲ (USP 1-May-2020)

FDA Guidance Documents

- *Guideline for the Determination of Residual Moisture in Dried Biological Products*, January 1990.
- *Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy*, March 1998. ▲https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm081670.pdf.▲ (USP 1-May-2020)
- *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*, ▲November 2006. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm078723.pdf.▲ (USP 1-May-2020)
- *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production*, October 2006. ▲https://www.fda.gov/downloads/drugs/guidances/ucm070287.pdf.▲ (USP 1-May-2020)
- *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*, April 2008. ▲https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm078694.pdf.▲ (USP 1-May-2020)
- ▲▲ (USP 1-May-2020)*Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*, ▲January 2011. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm243392.pdf▲ (USP 1-May-2020)

National and International Regulatory Documents

- ICH Q5A(R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q5D: Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- NIH Guidelines for Research Involving Recombinant ▲or Synthetic Nucleic Acid▲ (USP 1-May-2020) Molecules. ▲Available at: https://osp.od.nih.gov/biotechnology/nih-guidelines/.▲ (USP 1-May-2020)

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⟨1048⟩ QUALITY OF BIOTECHNOLOGICAL PRODUCTS: ANALYSIS OF THE EXPRESSION CONSTRUCT IN CELLS USED FOR PRODUCTION OF r-DNA DERIVED PROTEIN PRODUCTS[1]

I. INTRODUCTION

This document presents guidance regarding the characterization of the expression construct for the production of recombinant DNA (r-DNA) protein products in eukaryotic and prokaryotic cells. The document is intended to describe the types of information that are considered valuable in assessing the structure of the expression construct used to produce r-DNA derived proteins. The document is not intended to cover the entire quality aspect of r-DNA derived medicinal products.

The expression construct is defined as the expression vector containing the coding sequence of the recombinant protein. Segments of the expression construct should be analyzed using nucleic acid techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the final product. Analysis of the expression construct at the nucleic acid level should be considered as part of the overall evaluation of quality, taking into account that this testing only evaluates the coding sequence of a recombinant gene and not the translational fidelity nor other characteristics of the recombinant protein, such as secondary structure, tertiary structure, and posttranslational modifications.

II. RATIONALE FOR THE ANALYSIS OF THE EXPRESSION CONSTRUCT

The purpose of analyzing the expression construct is to establish that the correct coding sequence of the product has been incorporated into the host cell and is maintained during culture to the end of production. The genetic sequence of recombinant proteins produced in living cells can undergo mutations that could alter the properties of the protein with potential adverse consequences to patients. No single experimental approach can be expected to detect all possible modifications to a protein. Protein analytical techniques can be used to assess the amino acid sequence of the protein and structural features of the expressed protein due to posttranslational modifications such as proteolytic processing, glycosylation, phosphorylation, and acetylation. Data from nucleic acid analysis may be useful because protein analytical methods may not detect all changes in protein structure resulting from mutations in the sequence coding for the recombinant protein. The relative importance of nucleic acid analysis and protein analysis will vary from product to product.

Nucleic acid analysis can be used to verify the coding sequence and the physical state of the expression construct. The nucleic acid analysis is performed to ensure that the expressed protein will have the correct amino acid sequence, but is not intended to detect low levels of variant sequences. Where the production cells have multiple integrated copies of the expression construct, not all of which may be transcriptionally active, examination of the transcription product itself by analysis of m-RNA or c-DNA may be more appropriate than analysis of genomic DNA. Analytical approaches that examine a bulk population of nucleic acids, such as those performed on pooled clones or material amplified by the polymerase chain reaction, may be considered as an alternative to approaches that depend on selection of individual DNA clones. Other techniques could be considered that allow for rapid and sensitive confirmation of the sequence coding for the recombinant protein in the expression construct.

The following sections describe information that should be supplied regarding the characterization of the expression construct during the development and validation of the production system. Analytical methodologies should be validated for the intended purpose of confirmation of sequence. The validation documentation should, at a minimum, include estimates of the limits of detection for variant sequences. This should be performed for either nucleic acid or protein sequencing methods. The philosophy and recommendations for analysis expressed in this document should be reviewed periodically to take advantage of new advances in technology and scientific information.

III. CHARACTERIZATION OF THE EXPRESSION SYSTEM

A. Expression Construct and Cell Clone Used to Develop the Master Cell Bank (MCB)

The manufacturer should describe the origin of the nucleotide sequence coding for the protein. This should include identification and source of the cell from which the nucleotide sequence was originally obtained. Methods used to prepare the DNA coding for the protein should be described.

The steps in the assembly of the expression construct should be described in detail. This description should include the source and function of the component parts of the expression construct, e.g., origins of replication, antibiotic resistance genes,

[1] This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 29, 1995. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on February 23, 1996 (61 FR 7006) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on the production of r-DNA derived protein products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the "Regulatory Guidance" section).

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promoters, enhancers, and whether or not the protein is being synthesized as a fusion protein. A detailed component map and a complete annotated sequence of the plasmid should be given, indicating those regions that have been sequenced during the construction and those taken from the literature. Other expressed proteins encoded by the plasmid should be indicated. The nucleotide sequence of the coding region of the gene of interest and associated flanking regions that are inserted into the vector, up to and including the junctions of insertion, should be determined by DNA sequencing of the construct.

A description of the method of transfer of the expression construct into the host cell should be provided. In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail.

B. Cell Bank System

Production of the recombinant protein should be based on well-defined MCB and Working Cell Banks (WCB). A cell bank is a collection of ampules of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells. The MCB is generally derived from the selected cell clone containing the expression construct. The WCB is derived by expansion of one or more ampules of the MCB. The cell line history and production of the cell banks should be described in detail, including methods and reagents used during culture, in vitro cell age, and storage conditions. All cell banks should be characterized for relevant phenotypic and genotypic markers, which could include the expression of the recombinant protein or presence of the expression construct.

The expression construct in the MCB should be analyzed as described below. If the testing cannot be carried out on the MCB, it should be carried out on each WCB.

Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for copy number, for insertions or deletions, and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells retaining the expression construct should be determined.

The protein coding sequence for the recombinant protein product of the expression construct should be verified. For extrachromosomal expression systems, the expression construct should be isolated and the nucleotide sequence encoding the product should be verified without further cloning. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies. Alternatively, the nucleic acid sequence encoding the product could be verified by techniques such as sequencing of pooled c-DNA clones or material amplified by the polymerase chain reaction. The nucleic acid sequence should be identical, within the limits of detection of the methodology, to that determined for the expression construct as described in section III.A., and should correspond to that expected for the protein sequence.

C. Limit for In Vitro Cell Age for Production

The limit for in vitro cell age for production should be based on data derived from production cells expanded under pilot plant-scale or full-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could be used to prepare the production cells with appropriate justification.

The expression construct of the production cells should be analyzed once for the MCB as described in section III.B., except that the protein coding sequence of the expression construct in the production cells could be verified by either nucleic acid testing or analysis of the final protein product. Increases in the defined limit for in vitro cell age for production should be supported by data from cells that have been expanded to an in vitro cell age that is equal to or greater than the new limit for in vitro cell age.

IV. CONCLUSION

The characterization of the expression construct and the final purified protein are both important to ensure the consistent production of a r-DNA derived product. As described above, analytical data derived from both nucleic acid analysis and evaluation of the final purified protein should be evaluated to ensure the quality of a recombinant protein product.

GLOSSARY

Expression Construct: The expression vector that contains the coding sequence of the recombinant protein and the elements necessary for its expression.

Flanking Control Regions: Noncoding nucleotide sequences that are adjacent to the 5′ and 3′ end of the coding sequence of the product that contain important elements that affect the transcription, translation, or stability of the coding sequence. These regions include, e.g., promoter, enhancer, and splicing sequences, and do not include origins of replication and antibiotic resistance genes.

Integration Site: The site where one or more copies of the expression construct is integrated into the host cell genome.

In Vitro Cell Age: Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time in culture, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Master Cell Bank (MCB): An aliquot of a single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the MCB unless justified.

Pilot Plant Scale: The production of a recombinant protein by a procedure fully representative of and simulating that to be applied on a full commercial manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

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Relevant Genotypic and Phenotypic Markers: Those markers permitting the identification of the strain of the cell line that should include the expression of the recombinant protein or presence of the expression construct.

Working Cell Bank (WCB): The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

⟨1049⟩ QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS[1]

INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the product's potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

SCOPE OF THE ANNEX (2)

The guidance stated in this annex to "Stability Testing of New Drug Substances and Products" applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the "Glossary" in "Stability Testing of New Drug Substances and Products." However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

[1] This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 20, 1995. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the *Federal Register* on July 10, 1996 (61 FR 36466) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on stability testing of biotechnological/biological products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the "Regulatory Guidance" section).

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SELECTION OF BATCHES (4)

Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with acommitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

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STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable in vivo or in vitro quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, in vitro tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

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Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

STORAGE CONDITIONS (6)

Temperature (6.1)

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

Humidity (6.2)

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

Accelerated and Stress Conditions (6.3)

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

Light (6.4)

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

Container/Closure (6.5)

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

Stability after Reconstitution of Freeze-Dried Product (6.6)

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

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TESTING FREQUENCY (7)

The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

GLOSSARY (10)

Conjugated Product: A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product: A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products, some degradation products may be active.

Impurity: Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Intermediate: For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.

Manufacturing Scale Production: Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale: The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

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⟨1050⟩ VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect), and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document, the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterized cell banks. It covers products derived from in vitro cell culture, such as interferons, monoclonal antibodies, and recombinant deoxyribonucleic acid (DNA)-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown in vivo as ascites. In this latter case, special considerations apply and additional information on testing cells propagated in vivo is contained in *Appendix 1*. Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

(1) Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;

(2) Assessing the capacity of the production processes to clear infectious viruses;

(3) Testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to describe a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the MCB by several routes such as: (1) Derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

B. Adventitious Viruses That Could Be Introduced During Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: (1) Use of contaminated biological reagents such as animal serum components; (2) use of a virus for the induction of expression

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of specific genes encoding a desired protein; (3) use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used for Production

Table 1 shows examples of virus tests to be performed once only at various cell levels, including MCB, WCB, and cells at the limit of in vitro cell age used for production.

Table 1. Examples of Virus Tests to be Performed Once At Various Cell Levels

	MCB	WCB[1]	Cells at the Limit[2]
Tests for Retroviruses and Other Endogenous Viruses			
Infectivity	+	–	+
Electron microscopy[3]	+[3]	–	+[3]
Reverse transcriptase[4]	+[4]	–	+[4]
Other virus-specific tests[5]	as appropriate[5]	–	as appropriate[5]
Tests for Nonendogenous or Adventitious Viruses			
In vitro Assays	+	?[6]	+
In vivo Assays	+	–[6]	+
Antibody production tests[7]	+[7]	–	–
Other virus-specific tests[8]	+[8]	–	–

[1] See text—section III.A.2.
[2] Cells at the limit: Cells at the limit of in vitro cell age used for production (See text—section III.A.3.).
[3] May also detect other agents.
[4] Not necessary if positive by retrovirus infectivity test.
[5] As appropriate for cell lines which are known to have been infected by such agents.
[6] For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB's subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells at the limit of in vitro cell age.
[7] e.g., MAP, RAP, HAP—usually applicable for rodent cell lines.
[8] e.g., tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate.

1. MASTER CELL BANK

Extensive screening for both endogenous and nonendogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or nonhuman primate in origin, tests should be performed in order to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for nonendogenous viruses should include in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

2. WORKING CELL BANK

Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB. When appropriate nonendogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of in vitro cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

3. CELLS AT THE LIMIT OF IN VITRO CELL AGE USED FOR PRODUCTION

The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. The

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performance of suitable tests (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. *Table 2* outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or nonhuman primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus

Test	Test Article	Detection Capability	Detection Limitation
Antibody production	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system
In vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
In vitro virus screen for:		Broad range of viruses pathogenic for humans	Agents failing to replicate or product diseases in the test system
1. Cell bank characterization	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
2. Production screen	2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM on:		Virus and virus-like particles	Qualitative assay with assessment of identity
1. Cell substrate	1. Viable cells		
2. Cell culture supernatant	2. Cell-free culture supernatant		
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system
Cocultivation	Viable cells	Infectious retroviruses	RV failing to replicate
1. Infectivity endpoint			1. See above under RV infectivity
2. TEM endpoint			2. See above under TEM[1]
3. RT endpoint			3. See above under RT
PCR (Polymerase chain reaction)	Cells, culture fluid and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious.

[1] In addition, difficult to distinguish test article from indicator cells.

1. TESTS FOR RETROVIRUSES

For the MCB and for cells cultured up to or beyond the limit of in vitro cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Induction studies have not been found to be useful.

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2. IN VITRO ASSAYS

In vitro tests are carried out by the inoculation of a test article (see *Table 2*) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

3. IN VIVO ASSAYS

A test article (see *Table 2*) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

4. ANTIBODY PRODUCTION TESTS

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see *Table 2*) into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in *Table 3*.

Table 3. Virus Detected in Antibody Production Tests

MAP	HAP	RAP
Ectromelia Virus[2, 3]	Lymphocytic Choriomeningitis Virus (LCM)[1, 3]	Hantaan Virus[1, 3]
Hantaan Virus[1, 3]	Pneumonia Virus of Mice (PVM)[2, 3]	Kilham Rat Virus (KRV)[2, 3]
K Virus[2]	Reovirus Type 3 (Reo3)[1, 3]	Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]
Lactic Dehydrogenase Virus (LDM)[1, 3]	Sendai Virus[1, 3]	Pneumonia Virus of Mice (PVM)[2, 3]
Lymphocytic Choriomeningitis Virus (LCM)[1, 3]	SV5	Rat Coronavirus (RCV)[2]
Minute Virus of Mice[2, 3]		Reovirus Type 3 (Reo3)[1, 3]
Mouse Adenovirus (MAV)[2, 3]		Sendai Virus[1, 3]
Mouse Cytomegalovirus (MCMV)[2, 3]		Sialoacryoadenitis Virus (SDAV)[2]
Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]		Toolan Virus (HI)[2, 3]
Mouse Hepatitis Virus (MHV)[2]		
Mouse Rotavirus (EDIM)[2, 3]		
Pneumonia Virus of Mice (PVM)[2, 3]		
Polyoma Virus[2]		
Reovirus Type 3 (Reo3)[1, 3]		
Sendai Virus[1, 3]		
Thymic Virus[2]		

[1] Viruses for which there is evidence of capacity for infecting humans or primates.
[2] Viruses for which there is no evidence of capacity for infecting humans.
[3] Virus capable of replicating in vitro in cells of human or primate origin.

C. Acceptability of Cell Lines

It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, the action plan recommended for manufacture is described in section V. of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents

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one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least three lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. In vitro screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterizing the clearance of nonspecific "model" viruses (described later). Definitions of "relevant," specific, and nonspecific "model" viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. When a manufacturing process is characterized for robustness of clearance using nonspecific "model" viruses, particular attention should be paid to nonenveloped viruses in the study design. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described in section VI. below.

Table 4 presents an example of an action plan in terms of process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterization of clearance using nonspecific "model" viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the regulatory authorities. With Cases C, D, and E, it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk

	Case A	Case B	Case C[2]	Case D[2]	Case E[2]
Status					
Presence of virus[1]	–	–	–	–	(+)[3]
Virus-like particles[1]	–	–	–	–	(+)[3]
Retrovirus-like particles[1]	–	+	–	–	(+)[3]
Virus identified	not applicable	+	+	+	–
Virus pathogenic for humans	not applicable	–[4]	–[4]	+	unknown
Action					
Process characterization of viral clearance using non-specific "model" viruses	yes[5]	yes[5]	yes[5]	yes[5]	yes[7]
Process evaluation of viral clearance using "relevant" or specific "model" viruses	no	yes[6]	yes[6]	yes[6]	yes[7]

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Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk *(continued)*

	Case A	Case B	Case C[2]	Case D[2]	Case E[2]
Test for virus in purified bulk	not applicable	yes[8]	yes[8]	yes[8]	yes[8]

[1] Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.

[2] The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.

[3] Virus has been observed by either direct or indirect methods.

[4] Believed to be nonpathogenic.

[5] Characterization of clearance using nonspecific "model" viruses should be performed.

[6] Process evaluation for "relevant" viruses or specific "model" viruses should be performed.

[7] See text under Case E.

[8] The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

Case A

Where no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or in the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific "model" viruses as previously stated.

Case B

Where only a rodent retrovirus (or a retrovirus-like particle that is believed to be nonpathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific "model" virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as Chinese hamster ovary (CHO), C127, baby hamster kidney (BHK), and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk. Studies with nonspecific "model" viruses, as in Case A, are appropriate.

Case C

When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans (such as those identified by footnote 2 in *Table 3*, except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, "relevant" or specific "model" viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or "relevant" or specific "model") viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case D

Where a known human pathogen, such as those indicated by footnote 1 in *Table 3*, is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, "relevant" and/or specific "model" viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case E

When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.

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VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterization of due virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterized cell lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see section VI.B.5.).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale, which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses exhibiting a range of biochemical and biophysical properties that are not known or expected to be present is to characterize the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C.). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value need not be achieved.

A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterization study and the guidance provided in this document.

1. "RELEVANT" VIRUSES AND "MODEL" VIRUSES

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: "Relevant" viruses, specific "model" viruses, and nonspecific "model" viruses.

"Relevant" viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a "relevant" virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown in vitro to sufficiently high titers), a specific "model" virus should be used as a substitute. An appropriate specific "model" virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in *Appendix 2* and *Table A-1*.

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2. OTHER CONSIDERATIONS

Additional points to be considered are as follows:
(a) Viruses which can be grown to high titer are desirable, although this may not always be possible.
(b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.
(c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

1. FACILITY AND STAFF

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

2. SCALED-DOWN PRODUCTION SYSTEM

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations that cannot be avoided should be discussed with regard to their influence on the results.

3. ANALYSIS OF STEP-WISE ELIMINATION OF VIRUS

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (*Appendix 4*).

4. DETERMINING PHYSICAL REMOVAL VERSUS INACTIVATION

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

5. INACTIVATION ASSESSMENT

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast "phase 1" and a slow "phase 2." The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a "relevant" virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific "model" viruses are used or when specific "model" viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid

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to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

6. FUNCTION AND REGENERATION OF COLUMNS

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

7. SPECIFIC PRECAUTIONS

(a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.
(b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.
(c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as the dilution, concentration, filtration or storage of samples before titration.
(d) The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.
(e) Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.
(f) Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.
(g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.
(h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.
(i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies; Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See *Appendix 5* for calculation of virus reduction factors and *Appendix 6* for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:
(i) The appropriateness of the test viruses used;
(ii) The design of the clearance studies;
(iii) The log reduction achieved;
(iv) The time dependence of inactivation;
(v) The potential effects of variation in process parameters on virus inactivation/removal;
(vi) The limits of assay sensitivities;
(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well-designed separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

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An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 $\log_{10}$ or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.
2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.
3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 $\log_{10}$), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.
4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 $\log_{10}$ infectious units per milliliter (mL) by a factor of 8 $\log_{10}$ leaves zero $\log_{10}$ per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.
5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.
6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see *Appendix 3*).

F. Reevaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

A. Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;

B. Assessment of risk by determination of the human tropism of the contaminants;

C. Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;

D. Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and

E. Performance of studies which assess virus inactivation and removal.

GLOSSARY

Adventitious Virus: See *Virus*.

Cell Substrate: Cells used to manufacture product.

Endogenous Virus: See *Virus*.

Inactivation: Reduction of virus infectivity caused by chemical or physical modification.

In Vitro Cell Age: A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

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Master Cell Bank (MCB): An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the original MCB, unless justified.

Minimum Exposure Time: The shortest period for which a treatment step will be maintained.

Nonendogenous Virus: See *Virus*.

Process Characterization of Viral Clearance: Viral clearance studies in which nonspecific "model" viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

Process Evaluation Studies of Viral Clearance: Viral clearance studies in which "relevant" and/or specific "model" viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

Production Cells: Cell substrate used to manufacture product.

Unprocessed Bulk: One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

Virus: Intracellularly replicating infectious agents that are potentially pathogenic, possess only a single type of nucleic acid (either ribonucleic acid (RNA) or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

Adventitious Virus: Unintentionally introduced contaminant virus.

Endogenous Virus: Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

Nonendogenous Virus: Virus from external sources present in the MCB.

Nonspecific Model Virus: A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the purification process.

Relevant Virus: Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

Specific Model Virus: Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

Viral Clearance: Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

Virus-like Particles: Structures visible by electron microscopy which morphologically appear to be related to known viruses.

Virus Removal: Physical separation of virus particles from the intended product.

Working Cell Bank (WCB): The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

APPENDICES

Appendix 1: Products Derived from Characterized Cell Banks Which Were Subsequently Grown In Vivo

For products manufactured from fluids harvested from animals inoculated with cells from characterized banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in *Table 3*, should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum, and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in section IV. of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as in vivo testing in adult and suckling mice.

Appendix 2: The Choice of Viruses for Viral Clearance Studies

A. EXAMPLES OF USEFUL "*Model*" *Viruses:*

1. Nonspecific "model" viruses representing a range of physico-chemical structures:

• SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, nonenveloped viruses;

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- a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
- a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

These viruses are examples only and their use is not mandatory.

2. For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

B. EXAMPLES OF VIRUSES THAT HAVE BEEN USED IN VIRAL CLEARANCE STUDIES

Several viruses that have been used in viral clearance studies are listed in *Table A-1*. However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers are invited to consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

Table A-1. Examples of Viruses Which Have Been Used in Viral Clearance Studies

Virus	Family	Genus	Natural Host	Genome	Env	Size (nm)	Shape	Resistance[1]
Vesicular Stomatitis Virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70 × 150	Bullet	Low
Parainfluenza Virus	Paramyxo	Paramyxovirus	Various	RNA	yes	100–200	Pleo/Spher	Low
MuL V	Retro	Type C oncovirus	Mouse	RNA	yes	80–110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60–70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50–70	Pleo/Spher	Low
Pseudo-rabies Virus	Herpes		Swine	DNA	yes	120–200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25–30	Icosahedral	Med
Encephalomyocarditis Virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	no	25–30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	RNA	no	60–80	Spherical	Med
SV 40	Papova	Polyomavirus	Monkey	DNA	no	40–50	Icosahedral	Very high
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine	DNA	no	18–24	Icosahedral	Very high

[1] Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory.

Appendix 3: Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately $0.5 \log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses. If the 95 percent confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95 percent confidence limits for the reduction factor are

$$\pm\sqrt{S^2 + a^2}$$

Appendix 4: Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

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$$p = ((V-v)/V)^n$$

where V (L) is the overall volume of the material to be tested; v (L) is the volume of the sample; and n is the absolute number of infectious particles statistically distributed in V.

If V >> v, this equation can be approximated by the Poisson distribution:

$$p = e^{-cv}$$

where c is the concentration of infectious particles per L.

$$\text{or, } c = \ln p/-v$$

As an example, if a sample volume of 1 mL is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

c	10	10	1,000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

Appendix 5: Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material: vol v′; titer $10^{a'}$;
virus load: $(v')(10^{a})$,
Final material: vol v″; titer $10^{a''}$;
virus load: $(v'')(10^{a''})$,
the individual reduction factors Ri are calculated according to
$10^{Ri} = (v')(10^{a'}) / (v'')(10^{a''})$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

Appendix 6: Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

I. Assumptions

Measured or estimated concentration of virus in cell culture harvest = 10^6/mL
Calculated viral clearance factor = > 10^{15}
Volume of culture harvest needed to make a dose of product = 1 L (10^3 mL)

II. Calculation of Estimated Particles/Dose

$$\frac{(10^6 \text{ virus units/mL}) \times (10^3 \text{ mL/dose})}{\text{Clearance factor} > 10^{15}}$$

$$= \frac{10^9 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}}$$

$$= < 10^{-6} \text{ particles/dose}$$

Therefore, less than one particle per million doses would be expected.

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⟨1050.1⟩ DESIGN, EVALUATION, AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

INTRODUCTION

This chapter is a companion document to *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩, which was adapted, essentially unchanged, from the International Conference on Harmonization Q5A. This chapter provides users with practical guidance regarding the design, evaluation, and characterization of viral clearance procedures. The chapter scope is the same as that described in ⟨1050⟩ and covers biotechnology products for human use that are derived from cell lines of human or animal origin. Viral clearance studies performed according to the principles outlined in this chapter will provide meaningful data about the ability of the overall production and purification processes to remove or inactivate a broad spectrum of viral types that may affect the safety of biotechnology-derived products. [NOTE—The *Glossary* contains definitions of terms used in this chapter that are not already defined in ⟨1050⟩.]

The regulations for licensing biotechnology products stipulate that cell banks, biologically derived raw materials, and bulk harvest must be controlled and tested for viral safety; however, many of the viral detection approaches that can be used have inherent limitations. In addition, certain detection methods may be so specific for a particular virus that they may fail to detect viral variants (e.g., noncytopathic strains, or non-laboratory-adapted wild-type strains, or mutated variants). Some newly emerging viruses may be missed. Finally, detection methods typically are limited by their sensitivities, and infectious viruses at a low titer may go undetected. Viral contamination of manufacturing processes can come from many sources, including cell substrates and raw materials of biological origin (such as cell culture supplements and other production raw materials), and contamination during production. In addition to the required viral safety testing of these materials, manufacturers must evaluate the effectiveness of downstream purification process steps, which together must remove or inactivate any viruses potentially present (see ⟨1050⟩). USP encourages readers to maintain and keep current best practices in the field by reviewing current peer-reviewed publications, regulatory guidances, and key opinion leaders and organizations.

As suggested in the U.S. Food and Drug Administration "Points to Consider" documents (*Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use* and *Draft Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*), as well as ⟨1050⟩, a multi-step approach is typically used to ensure viral safety. Clearance studies can generally be considered generic or modular. As described in the FDA's *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use*, a generic clearance study is one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model monoclonal antibody (mAb) in which the data may be extrapolated to other mAbs following the same purification and virus removal/inactivation scheme as the model mAb. A modular clearance study demonstrates virus removal or inactivation of individual steps during the purification process (column chromatography, filtration, pasteurization, solvent/detergent, low pH, and others) so that each module in the purification scheme may be studied independently of the other modules. If necessary, an alternative model mAb can be used to demonstrate viral clearance in different modules. Identical clearance modules can be extrapolated to the product mAb. This chapter complements these documents by providing users strategies to perform and assess viral clearance.

GOALS AND PRINCIPLES OF VIRAL CLEARANCE STUDIES

A viral clearance study should evaluate the ability of the overall purification process to remove or inactivate a broad spectrum of virus types, including viruses that are known to contaminate or have the potential to contaminate the raw materials, and those that can be introduced during manufacturing. Viral clearance studies typically involve substudies performed on specific and suitable individual steps of the manufacturing and purification process. The studies should be performed in a manner that generates quantitative data, allowing for identification of effective clearance steps and estimation of viral reduction factors (VRFs, also known as log reduction factors, LRF, or log reduction values, LRV). The VRFs of an individual clearance step represent the ratio of the viral load in the pretreatment material (used to challenge the clearance step) to the viral load in the post-treatment material. The VRFs derived from specific process steps are used to evaluate the overall capacity of the entire production process to remove or inactivate process-specific or nonspecific viruses.

One of the key goals of a manufacturing purification process is to achieve maximal viral clearance without compromising product quality. Critical attributes of strategic viral clearance steps in the manufacturing process must be characterized. In the context of viral clearance, robustness has two main components: 1) the ability of process steps to remove and/or inactivate viruses under worst-case conditions or over a wide operational range for parameters (e.g., temperature, protein concentration, pressure, flow rate, conductivity, and pH), and 2) the ability of process steps to consistently remove or inactivate nonspecific viruses that possess a broad spectrum of physical and chemical resistance characteristics (e.g., pH, heat, solvent/detergent treatment, or filtration). A validated process that provides robust viral clearance should establish the VRF achievable for a panel of "relevant" or "model" viruses (see next section for definitions). The validated process also should demonstrate robustness of dedicated inactivation or removal steps for which critical operational parameters that could affect viral clearance are well established. Therefore, a demonstration of viral clearance robustness provides confidence that the manufacturing process can remove or inactivate potential viral contaminants.

Viral clearance study protocols begin with an overall action plan and design of experiments. Chapter ⟨1050⟩ (see *Section V* and *Table 4*) describes five major action plans (*Cases A–E*) and can serve as a guide. Cell substrates categorized in *Cases C, D,* and *E* represent special cases, and manufacturers who use these substrates should discuss study design with the applicable regulatory authorities. Unique issues for each production process must be considered on a case-by-case basis. These issues may relate to the starting materials, production process, product, and intended use of the product. The overall action plan dictates the choice of viruses used in the viral clearance study, i.e., the study must include relevant, specific, and nonspecific model

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viruses. Further, the viral clearance study protocol may include the following elements: descriptions of the study facility and staff responsible for executing the study, the scaled-down purification models, justification of the appropriateness of the scaled-down model, and the study design. The study report may include these elements, as well as a stepwise analysis of calculated VRFs and the overall viral clearance capacity of the purification process. Viral clearance studies should be conducted in a well-documented, controlled manner that complies with current regulatory requirements.

CONSIDERATIONS FOR PERFORMANCE OF VIRAL CLEARANCE STUDIES

Selection of Viruses

The choice and number of viruses that may be used in a viral clearance study are dictated by the nature and origin of the production cell line, as well as the nature and origin of the animal-derived materials used in production and purification. In general, at least two viruses, one enveloped (typically a retrovirus, e.g., MuLV) and one nonenveloped (preferably a parvovirus, e.g., MVM), are used in the early clinical phases of product development, but three or more viruses may be used to generate data for registration-enabling studies. At least two orthogonal virus removal/inactivation steps (steps with different mechanisms of clearance) should be evaluated per virus. The reproducibility of an effective step should be assessed by performing at least two independent experiments, or reproducibility should be supported by the process development history (or experiences). Model viruses for process evaluation and process characterization studies should be similar to the virus that may contaminate the product; however, other viruses with a wide range of physical and chemical characteristics should also be examined. The latter is important for showing that the purification process is capable of inactivating or removing a wide variety of viruses, including newly emerging viruses (e.g., vesivirus 2117, circoviruses, or newly discovered animal and human parvoviruses) or unexpected contaminants (e.g., BVDV, MVM, epizootic hemorrhagic disease virus, and Cache valley virus). The manufacturer should justify the choice of viruses in accordance with the aims of the process evaluation and process characterization studies and the guidance provided in this document, as well as relevant regulatory guidelines.

Viruses used in clearance studies fall into three categories: relevant viruses, specific model viruses, and nonspecific model viruses. "Relevant viruses" are viruses used in process evaluation studies, which are either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. When a relevant virus is not available or when it is not well adapted for this purpose (e.g., it cannot be grown in vitro to a sufficiently high titer or cannot be detected using cell-based viral titration endpoints required for assessing inactivation), a "specific model virus" can be used as a substitute. An appropriate specific model virus may be a virus that is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus. For example, cell lines derived from rodents usually contain endogenous retroviral particles or retroviral-like particles that can be infectious or noninfectious. The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses present in products obtained from such cells should be determined. In the case of rodent-derived cells, one can use a murine leukemia virus as a model virus. Manufacturers using human-derived B-lymphocyte cells immortalized by infection with Epstein–Barr virus should demonstrate the ability of the manufacturing process to clear a herpesvirus. In this instance, pseudorabies virus or another herpesvirus can be used as a specific model virus.

Finally, when the goal is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general (i.e., to characterize the robustness of the clearance process), viral clearance studies can be performed with a panel of "nonspecific model viruses" that have a wide range of physical and chemical characteristics. Data obtained from studies with relevant or specific model viruses also can contribute to this assessment. It is not necessary to test all types of viruses; preference should be given to viruses with diverse physical and chemical properties that would be representative of a wide range of viral families. The results obtained for such viruses provide useful information about the ability of the production process to remove or inactivate viruses in general. *Table 1* gives examples of model viruses that represent a range of physicochemical properties for use in viral clearance studies, including those examples already described in ⟨1050⟩ (see *Table A-1*). Additional points to consider during viral selection are: 1) ability to grow the virus of interest to a sufficiently high titer, 2) ability to create stocks with minimal aggregates, 3) availability of a qualified/validated assay system for detection of the selected virus, 4) the selected viruses are not likely to pose a health hazard to personnel performing the study or the environment, and 5) potential to address new and emerging viruses.

Table 1. Examples of Viruses Used in Viral Clearance Studies for Biotechnology Products Derived from Cell Cultures

Virus	Family	Genus	Natural Host	Genome	Enveloped	Size (nm)	Shape	Resistance[a]
Adenovirus 5	Adeno	Mastadeno-virus	Human	DNA	No	70–90	Spherical	High
BVDV	Flavi	Pestivirus	Bovine	RNA	Yes	50–70	Pleomorphic/ spherical	Low
Cache valley virus	Bunya	Bunyavirus	Bovine, ovine	RNA	Yes	80–120	Spherical	Low
Encephalomyocarditis virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	No	25–30	Icosahedral	Medium
Feline calicivirus	Calici	Vesivirus	Feline	RNA	No	35–40	Icosahedral	High
Herpes simplex 1	Herpes	Alpha-herpesvirus	Human	DNA	Yes	120–200	Spherical	Medium
MuLV	Retro	Type C oncovirus	Mouse	RNA	Yes	80–110	Spherical	Low

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Table 1. Examples of Viruses Used in Viral Clearance Studies for Biotechnology Products Derived from Cell Cultures *(continued)*

Virus	Family	Genus	Natural Host	Genome	Enveloped	Size (nm)	Shape	Resistance[a]
Parainfluenza virus	Paramyxo	Paramyxovirus	Various	RNA	Yes	100–200	Pleomorphic/ spherical	Low
Parvoviruses (MVM, PPV, CPV, BPV)	Parvo	Parvovirus	Murine, porcine, canine, bovine	DNA	No	18–26	Icosahedral	Very High
Poliovirus sabin type 1	Picorna	Enterovirus	Human	RNA	No	25–30	Icosahedral	Medium
Porcine circovirus (PCV)	Circo	Circovirus	Porcine	DNA	No	15–20	Icosahedral	High
Pseudorabies virus	Herpes	—	Swine	DNA	Yes	120–200	Spherical	Medium
Reovirus 3	Reo	Orthoreovirus	Various	RNA	No	60–80	Spherical	Medium
Simian virus 5	Paramyxo	Rubulavirus	Simian	RNA	Yes	150–300	Pleomorphic/ spherical	Low
Sindbis virus	Toga	Alphavirus	Human	RNA	Yes	60–70	Spherical	Low
SV40	Papova	Polyomavirus	Monkey	DNA	No	40–50	Icosahedral	Very high
Vesicular stomatitis virus	Rhabdo	Vesiculovirus	Equine, bovine	RNA	Yes	70 × 150	Bullet	Low
West Nile virus	Flavi	Flavivirus	Avian	RNA	Yes	40–70	Pleomorphic/ spherical	Medium

[a] Resistance to physicochemical treatments based on studies of purification processes. Resistance is relative to the specific treatment, and it is used in the context of understanding the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only, their use is not mandatory, and the table is not exhaustive.

Process Clearance Capability

The viral clearance capability of a process is typically expressed as the logarithmic value of the virus reduction factor evaluated by the entire process. Although there are no specific requirements for the clearance capability that a process must achieve, the nature and origin of the cell type, raw materials used in production, as well as other factors may influence the need for greater or lower levels of viral clearance. For example, the target retroviral clearance capability for a CHO and NS0 cell manufacturing process could be 4–6 LRF of total-process retroviral clearance above the level found in the maximum therapeutic dose, based on the retroviral-like particle titer in the production bioreactor material. Minimum clearance capability for other types of viruses might include >4 LRF of clearance for additional enveloped viruses and >4 LRF of clearance for nonenveloped viruses. Specific clearance targets must be established and justified on a case-by-case basis. In general, for a single virus clearance step to be considered effective, 4 LRF or more of clearance must be demonstrated. In contrast, a clearance step demonstrating 1–3 LRF is considered a supportive step. The titer of the virus input and output for any given process step evaluated should include 95% confidence ($\alpha = 0.05$). A step can be supportive for some viruses and effective for others. When possible and as process knowledge allows, the analyst should perform viral clearance studies under expected worst-case conditions for the parameters defined as critical for that step (e.g., pH, temperature, and flow rate). Sufficient numbers of downstream purification process steps should be identified and evaluated so that the required overall clearance target can be reached.

Downstream Processing Steps for Viral Clearance

Downstream processing steps should employ different (orthogonal) mechanisms of viral clearance because consideration of multiple steps of the same or similar clearance mechanisms may lead to overestimation of the overall viral reduction capability. In general, a purification process should include at least one robust viral inactivation step and/or one robust viral removal step. For every purification step assessed, the mechanism of viral clearance should be described as inactivation, removal, or a combination of both; if both, the primary clearance mechanism should be identified. For inactivation steps, clearance studies should be planned in such a way that they can determine the kinetics of viral inactivation. Ideally, a manufacturing process includes steps that are dedicated to viral clearance; that is, they are present in the production or purification process specifically for the removal or inactivation of viruses. Examples of dedicated steps include solvent and detergent treatment, low-pH inactivation, and virus-removal filtration.

Qualification and Scale-Down of Purification Steps

Viral clearance studies using infectious virus spikes are not conducted at production scale in the manufacturing suite because this could contaminate the suite with viruses. Such studies should be performed in a segregated facility equipped for virological work and staffed with personnel who have virology expertise and familiarity with the operation of a scaled-down purification process. Each scaled-down step of the purification process must be qualified, i.e., found to be comparable to the full-scale production process by all relevant, measurable criteria. Comparability should be demonstrated using representative raw

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materials and intermediates from production, and equipment with process parameters through appropriate scale-down principles. The outputs should be measured with the appropriate analytical methods and statistical analyses.

In many instances, the monitored parameters are the same as those analyzed during performance of the actual manufacturing steps. For chromatography, the parameters should be representative of the respective clinical or commercial-scale manufacturing: [e.g., the column bed height, residence time, linear flow rate, chromatographic matrix, buffer composition (including pH, conductivity, and operating temperature), and product pool concentrations and composition]. Scaled-down and manufacturing-scale chromatography systems should produce similar elution profiles and step yields, and final product analytical profile (e.g., SEC-HPLC and/or SDS-PAGE analyses). Similar considerations apply for other types of procedures. Any unavoidable differences between scaled-down and manufacturing-scale procedures should be investigated to determine their potential influence on the viral clearance results.

Selection of Sampling Points

Sampling points for assessing viral inactivation should include the starting materials (virus-spiked process solutions and appropriate controls) as well as samples taken at several time points during the inactivation process. This approach allows the analyst to monitor the kinetics of viral inactivation. Sampling points for virus-removal steps, such as chromatography and filtration, should include the feed-stream process solution that will be applied to the step, as well as the resulting process pool that is then processed in the subsequent steps. Chromatographic fractions (e.g., flow-through, washes, pre-peak, peak, post-peak, strip) of the mainstream, as well as pre- and post-mainstream samples, typically are evaluated in studies that support registration-enabling studies. Analyzing such side fractions might also be helpful in cases where the mechanism of partitioning is not understood. Sampling points are discussed further in the section *Performance of Viral Spiking Studies*.

Selection of Assays for Viral Quantitation

Various types of viral quantitation assays can be used. Examples include infectivity assays such as tissue-culture infectious-dose assays and plaque quantitation assays. In addition, quantitative polymerase chain reaction (qPCR) assays detect and quantify the nucleic acids of both infectious and noninfectious viruses. These assays are described in *Virology Test Methods* ⟨1237⟩, *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, and *Nucleic Acid Based Techniques—Amplification* ⟨1127⟩. When using nucleic acid-based techniques (NAT), it is important to assure that the virus spike and the spiked samples to be analyzed do not contain significant amounts of free viral nucleic acids. The amount of free nucleic acids can be reduced by nuclease pretreatment of samples. It is important to characterize the impact of nuclease pretreatment on the virus spike since it could have a negative impact on the virus spike. Quantitative/quantal infectivity assays should have adequate sensitivity and reproducibility, and should be performed with a sufficient number of replicates to ensure adequate statistical validity of the results. All assays should include appropriate system suitability controls. Virus titration assays used in support of viral clearance studies must be shown to be suitable for this purpose by means of qualification or validation.

Sample Matrix Effects on Viral Quantitation Assays

When a virus is cleared by inactivation or removal (e.g., filtration or column chromatography) methods, the analysts must perform preliminary cytotoxicity and viral interference analyses to determine if their viral quantitation assays are sensitive to matrix components present in their samples. These studies should be performed before the spiking studies to confirm that the results of the viral clearance spiking studies are actually due to viral clearance and not to other intrinsic or extrinsic factors yielding a false-negative result. For example, some process intermediates may be cytotoxic to the detector (indicator) cells or may interfere with virus detection by a particular assay system. The process solutions used in these studies should be representative of those from a full-scale good manufacturing practice (GMP) manufacturing run. The dilution of the process sample solution that does not cause cytotoxicity or interference is determined and the process solution is diluted accordingly before storage or testing samples from that manufacturing step in the virus-spiking studies.

In a cytotoxicity evaluation, the cells used in the viral quantitation assay are exposed to a series of dilutions of the sample matrix solution for that process step. Then the cells are assessed for viability and changes in morphology that could interfere with evaluation of the indicator cells for viral cytopathic effects or plaques and hence could have an impact on assay performance. The dilution of the process sample solution that does not cause cytotoxicity is determined, and the process solution is diluted accordingly before storage or testing samples from that manufacturing step in the virus-spiking studies.

The study involves the following steps:

1. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus inactivation or filtration load using appropriate cell culture media as the diluent
2. Inoculate the different dilutions of virus inactivation or filtration load alone onto the indicator cells that will be used for each virus titration
3. Include appropriate buffer or media and cell controls
4. Identify the dilution of the virus inactivation load that does not exhibit measurable detector cell toxicity attributable to the load matrix

Process solutions also can interfere with virus detection by the in vitro cell-based viral infectivity assay system, either by inactivating the virus itself or by altering the indicator cells in a way that delays or prevents occurrence of a productive viral infection. To assess a process solution for viral interference, analysts prepare dilutions of the process solution and spike them with a known quantity of virus. Each dilution is subsequently titrated in the quantitative/quantal viral infectivity assay. If the difference between the known titer and the titer determined for the spiked solution in the assay (the viral interference titer) differ by more than the predetermined assay variability (typically ±0.5 log), then viral interference is suspected.

To assess the inactivation step load for viral interference, analysts should follow these steps:

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1. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus stock using appropriate cell culture media as the diluent. This will serve as the positive control for the virus titer
2. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus stock using the virus inactivation load as the diluent [NOTE—Information from the cytotoxicity study will determine the design of this step and the next one.]
3. Inoculate the dilution series onto the indicator cells
4. Include assay suitability controls (positive and negative) in the test
5. Identify the dilution of the virus inactivation load that does not result in significant changes in virus stock titer compared to the positive control virus titer

If qPCR assays are used to quantitate the virus, each process solution should be tested for interference and recovery with the nucleic acid extraction method used, as well as for interference with the qPCR reaction itself. Each process solution must be diluted to a level that does not cause interference. Although diluting a process solution to achieve acceptable assay performance is a commonly used strategy, this reduces the maximum demonstrable VRF for the clearance step under evaluation.

Effects of Storing and Freezing Viral Clearance Samples

If process solutions are used within their established hold times, stability studies should be performed to better understand the effect on the clearance step of holding or storing the solutions, or on the virus (e.g., aggregation). For example, bacterial growth or precipitation of proteins in the solutions may occur, particularly after prolonged storage.

If the samples from the spiking study are stored frozen before virus quantitation, analysts should perform stability studies using the viral infectivity result as an endpoint. Ideally, this should be done before the viral spiking study by spiking dilutions of each process solution with a known amount of virus and then freezing each sample. After specified times in frozen storage, the samples are removed, and the titer of each sample dilution is determined. If the difference between the known titer and the titer determined from the spiked frozen solution is greater than the assay variability (typically ±0.5 log), this indicates that viral infectivity has been compromised by frozen storage.

Qualification of Virus Stocks and Effects on Processing Steps

The virus stocks used in viral clearance studies are critical reagents. Each virus stock should have a traceable, certified source with full documentation of the controlled production procedures. Virus stocks should meet predetermined criteria for identity, purity (e.g., sterility, mycoplasma, and adventitious virus testing), infectious titer, stability at freezing temperatures, and minimal viral aggregates. Analysts should minimize the number of passages from the master or working viral bank in order to reduce the chance of mutation. Purity of virus preparations should be taken into account in cases when virus preparation impurities may influence the performance of a certain unit operation (e.g., virus filtration).

During qualification, analysts should obtain an initial virus titer for each virus stock. Ideally, analysts should independently assay viral stocks for titer on separate days using different passage numbers of the indicator culture cells that were used in the viral quantification assay. The resulting titers should agree within the predetermined variability of the assay (typically the expectation is ±0.5 log), and if so, the analyst can average the titers to determine the certified titer of the stock.

It may be advisable to perform a mock spiking study before the true spiking study. This mock spiking study evaluates the effects of the spiking virus matrix on the process step that is under evaluation. Mock spiking studies are important for all steps that will be tested for viral clearance, but they are particularly important for those steps that will be evaluated for the clearance of virus from a stock that contains impurities or additives (e.g., stabilizing protein). Mock spiking studies are conducted by adding the viral suspension matrix (including all components except the virus) to the process solution at a virus-spike to feed-stream-solution (load) ratio of NMT 10% (v/v) and then executing the processing step. The mock-spiked step is monitored for expected performance, and if results are different than expected, then reducing the virus:load spiking ratio may be helpful. This adjusted virus-spike to feed-stream-solution (load) ratio would be used in performing the actual clearance study for the processing step under study.

Performance of Viral Spiking Studies

Viral clearance studies are usually conducted either at a site where both the spiking and viral quantitation assays are performed (e.g., a biosafety testing laboratory) or at two different sites: one where the spiking studies are done and a second where the samples are quantitated. Sponsors and analysts must gain knowledge and control factors that can influence each manipulation that is not part of the manufacturing process, for example, freezing and thawing (see *Effects of Storing and Freezing Viral Clearance Samples*) as well as shipping conditions.

Before initiating a clearance study, analysts should prepare a well-documented study design that clearly defines the following: the steps that will be tested, the sampling plan for each step, sample identification, sample handling and storage, sample shipment if required, critical operating parameters, process scale, rationale for worst-case conditions (if they have been established), and the appropriate controls for each step. At a minimum, the samples tested should include those used to determine: 1) the spiking virus titer, 2) the spiking virus titer after freeze–thaw (if applicable), 3) the virus titer in the production solution before processing, and 4) the virus titer after processing. The analyst may need to collect and assay additional samples, depending on the step under test and the phase of product development.

In a typical biomanufacturing process, viral clearance can be accomplished by virus inactivation (e.g., pH treatment, heat treatment, or solvent and detergent treatment) or by virus removal (e.g., filtration or column chromatography). *Figures 1A* and *1B* show examples of experimental designs for virus inactivation, *Figure 2* shows virus removal by filtration, and *Figure 3* shows virus removal by column chromatography. These examples are intended for general reference and do not represent every specific condition. In developing their study designs, sponsors should consider their own process conditions in order to plan their own specific steps.

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The overall concept of viral clearance procedures can be expressed as follows:

Viral Clearance = Viral Inactivation + Viral Removal, where

Viral Removal = Removal by Filtration + Removal by Column Chromatography

VIRAL CLEARANCE BY VIRUS INACTIVATION

General considerations: Analysts should recognize that virus inactivation is not always a simple, first-order reaction. Typically, virus inactivation is complex and involves a fast initial phase followed by a slower phase. Thus, analysts should plan the inactivation process in such a way that samples are taken at different times in order to construct an inactivation time curve. Samples collected for inactivation studies should include the planned process time, and at a minimum, time zero; a time point or a suitable number of greater than zero but less than the minimum inactivating-agent exposure time; a time point equal to the minimum inactivating-agent exposure time; and in addition a time point beyond the minimum exposure time might be helpful in cases where there is a slow inactivation curve. Additional time points may be particularly important when there is no prior experience with virus inactivation kinetics.

When viral clearance by inactivation occurs, two scenarios generally are possible: 1) the inactivating agent is not present in the load material and inactivation is initiated by addition of the inactivating agent after virus spiking (*Figure 1A*); and 2) the load material already contains the inactivating agent before the virus spike, and inactivation is initiated by addition of the virus spike (*Figure 1B*). An example of the latter is a Protein A column eluate at low pH. Whenever possible, analysts should determine the initial virus load from the virus-spiked load material before they add an inactivating agent. However, this is not possible when the inactivating agent is already present in the load material at the start of the experiment. In these situations, the initial virus load can be calculated using the certified titer value of the spiking virus preparation and the virus:load spiking ratio. When viral inactivation occurs too rapidly to allow for plotting an inactivation time curve under normal process conditions, analysts should include appropriate controls to show that activity is indeed lost due to the inactivating agent present.

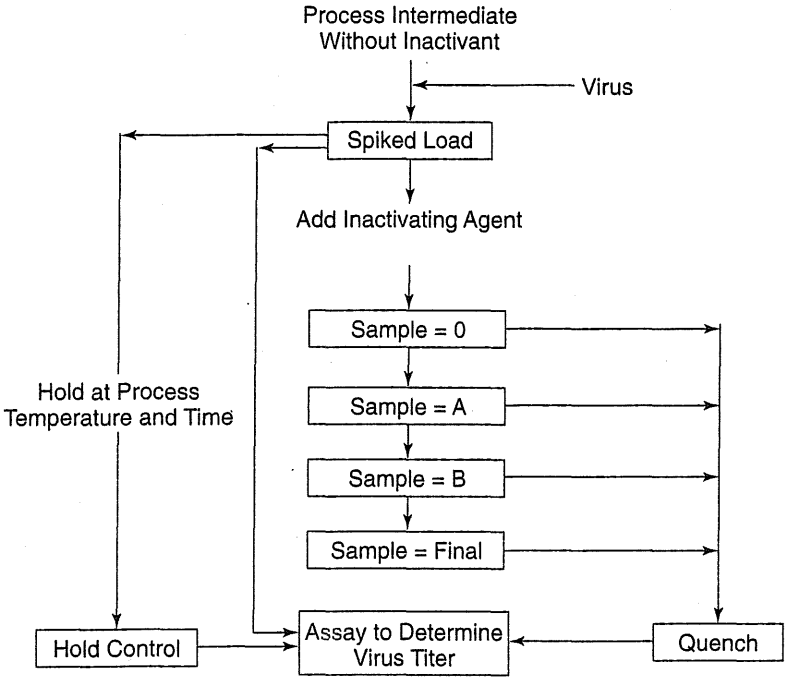

Figure 1A. Virus inactivation when load material does not contain an inactivating agent; the inactivating agent is added after viral spiking.

Study design: The general approach to the study design for assessing viral clearance by inactivation is outlined in *Figures 1A* and *1B*. In *Figure 1A*, the virus spike is added to the load material (process intermediate) before initiation of inactivation. The virus and load material are mixed well, and the spiked load and hold control samples are pulled. The spiked load should be tested immediately to determine virus titer. The actual titer of the spiked load (obtained experimentally) should be compared to the theoretical titer of the spiked load (obtained from the certified titer of the lot of virus used and the spiking ratio) to verify performance of virus detection assay and that the appropriate amount of virus was added to the load material. The hold control should be treated the same as the spiked load (i.e., prior to neutralization of the hold control, the sample should be pH adjusted in the same manner as the spiked load and then neutralized and spiked with virus). In addition, the hold control should remain at process temperature until the final sample in the experiment is pulled. The viral titer of the hold control then is assessed and compared to the actual titer of the spiked load to determine if any virus was inactivated in the presence of the load material over time and at the temperature of the inactivation study. This may be considered when determining the final VRF.

Inactivation should be initiated in a way that mimics the manufacturing process (e.g., with constant mixing) so that the inactivating agent (e.g., detergent, or acid utilized to lower pH) is mixed homogeneously into the solution as soon as possible. This minimizes the presence of localized high concentrations of the inactivating agent. If heat is used for inactivation, the heat

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should be applied in a way that mimics manufacturing (e.g., the ramp to target temperature should be identical) and controls should be kept unheated. However, the exact reproduction of addition (mixing) of inactivating agents or reproduction of a heating ramp can be very difficult at down-scale. In such cases it is advised to spike the virus directly into the inactivation agent-containing (or heated) material and to follow inactivation kinetics as described in *Figure 1B*. For control of the added virus, virus is spiked into process material without the inactivating agent or into unheated material.

Worst-case process conditions (e.g., pH, time, and temperature) should be applied if they have been established. Time points (samples) are pulled and the reaction is immediately quenched (e.g., by neutralization, dilution, or immediate cooling of the sample) so that further viral inactivation cannot occur. This allows the analyst to determine the kinetics of virus inactivation and then construct a virus inactivation versus time curve. Large-volume sampling can be applied to maximize clearance values.

In *Figure 1B*, the load material contains the inactivating agent and thus the addition of the virus spike starts the inactivation. In this scenario, it may not be possible to neutralize the process intermediate solution before the virus-spiking study because large fluctuations in pH may cause the protein product to fall out of solution. Also, the spiked load and hold control samples cannot be pulled from the virus-spiked load material, thus the control samples are pulled from a media or buffer control that does not contain the inactivating agent.

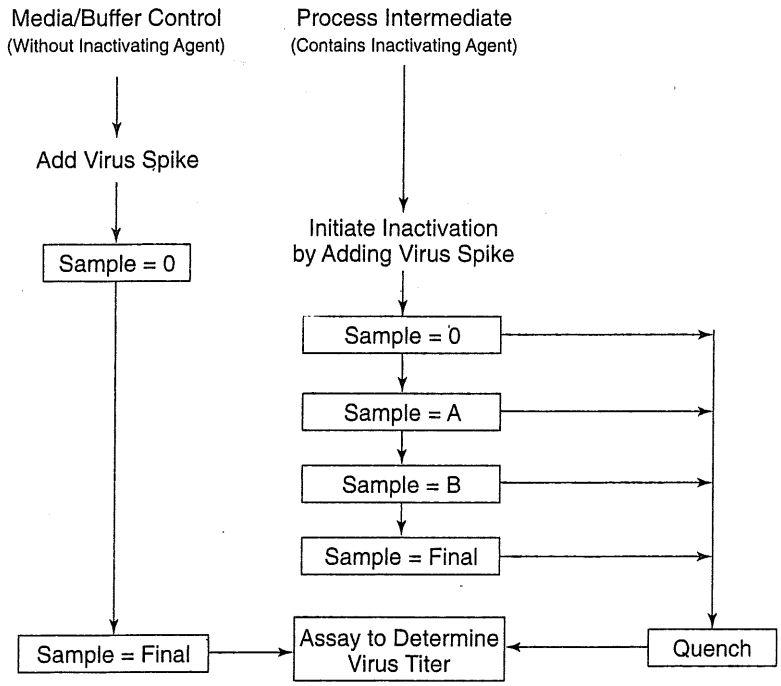

Figure 1B. Virus inactivation when the load material contains the inactivating agent before the virus spike; the addition of virus spike starts inactivation.

VIRAL CLEARANCE BY FILTRATION

Analysts must perform preliminary cytotoxicity and viral interference analyses when preparing to perform viral clearance by filtration (see *Sample Matrix Effects on Viral Quantitation Assays*). The viral filter load and pool dilution that will be used in the clearance study is that dilution shown not to cause cytotoxicity or interference.

Study design: The general approach to the study design for assessing viral clearance by filtration is outlined in *Figure 2*. Once the process intermediate is spiked with virus, the spiked load sample should be assayed immediately for virus titer. Virus spiking can be associated with aggregation effects. The aggregation status of a contaminating virus in the respective product intermediate is difficult to predict and therefore, as a worst-case approach, a mono-disperse virus preparation is advised for spiking. Filtration of virus before spiking (e.g., 0.45 µm or 0.22 µm) reduces larger virus aggregates from frozen virus stocks. A pure high-titer virus preparation can be helpful to reduce virus spike-induced effects influencing performance of virus filtration. The hold control sample should be maintained at processing conditions (temperature and time) and then assayed to evaluate the impact of these conditions, as well as the process intermediate components, on virus titer. Worst-case process conditions should be applied, if they have been established. The virus titers obtained from the spiked load and hold control should be within the experimental variability of the virus titration assay. If there is a negative impact on virus titer because of the processing conditions (temperature, time, and process intermediate composition), then an investigation should begin to understand the process. If warranted, the final VRF may need to be adjusted to account for the impact of hold conditions on viral preparations. However, this approach might underestimate the removal capacity from virus filtration. Alternatively, use of another detection method (e.g., NAT) or another, more stable, model virus can be discussed.

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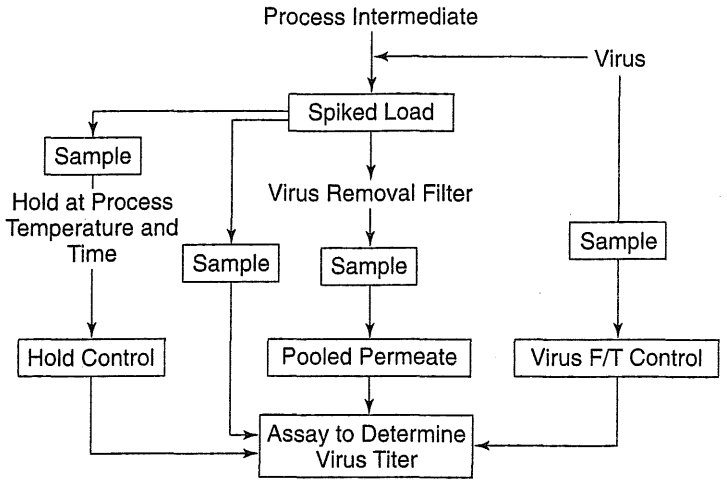

Figure 2. Clearance of virus by filtration methods.

Appropriate pre- and post-filtration controls should be used to assess removal of the virus spike by the prefiltration step. Samples are evaluated using virus titration assays. Test samples may require neutralization to appropriate pH levels for cell-culture-based titration assays. If a 0.22-µm or 0.45-µm prefilter is used during production in line with the virus-reduction filter, spiking could be performed on the intermediate before prefiltration. However, a sample should be taken from the stage after prefiltration in order to determine the VRF. Alternatively, the virus spike can be added to the intermediate after this prefiltration (using a similarly filtered virus spike) in order to determine virus reduction caused by the virus filter. To gain additional understanding of the virus clearance process, analysts can take samples at various time points throughout the run. In the event that samples are stored frozen prior to virus quantitation, a virus freeze/thaw (F/T) control is suggested to determine if there is any impact of the F/T cycle on virus titer (see also *Effects of Storing and Freezing Viral Clearance Samples*).

VIRAL CLEARANCE BY COLUMN CHROMATOGRAPHY

General considerations: For registration-enabling studies, analysts should investigate the distribution of the virus load among different chromatography fractions. Generally, analysts add virus to the starting intermediate (feed stream or load) for each step tested and determine the virus titer of the spiked load material and product pool spiked load material before and after the step. Fractions, in addition to the main product pool, may require testing at the later stages of product development (phase 3 trials and beyond). As is usual for viral clearance, these studies should be performed in duplicate. With column chromatography, the ability of the columns as well as other devices to clear virus may increase or decrease after repeated use. Evidence of consistent viral clearance after multiple uses may provide support for repeated use of such columns. Typically, this evidence is obtained when analysts compare clearance on new resin versus resin that has been cycled to or slightly beyond the targeted column lifetime, i.e., the number of times the column will be used in commercial manufacturing. For registration-enabling studies, data should also be provided to show that any virus potentially retained by the resin (carry-over) will be adequately inactivated or removed by cleaning procedures before reuse of the column.

For each manufacturing step assessed for viral clearance, the probable mechanism of reduction in viral infectivity should be known and described as virus inactivation or virus removal, or both. In some situations, it may be necessary to distinguish between removal and inactivation. For example, a column chromatography step that physically separates virus from product also may use a buffer capable of inactivating virus. In such situations, it may be possible to combine the use of viral infectivity assays with NAT assays to measure the individual contributions of the inactivation and removal mechanisms. Dissection of each step to determine the relative contribution of each mechanism of clearance allows for a thorough understanding of how viral clearance is achieved. This understanding may help to identify critical variables in each clearance step that should be controlled to support reproducible clearance. The testing of each critical variable (when it is well understood) at worst-case conditions helps to evaluate the process robustness of the step.

To measure virus clearance by a column chromatography system, analysts must perform preliminary cytotoxicity and viral interference analyses (see *Sample Matrix Effects on Viral Quantitation Assays* for details). The column load dilution that will be used in the clearance study is that dilution shown not to cause cytotoxicity or interference. [NOTE—Interference and cytotoxicity studies also can be performed on collected fractions, if warranted.]

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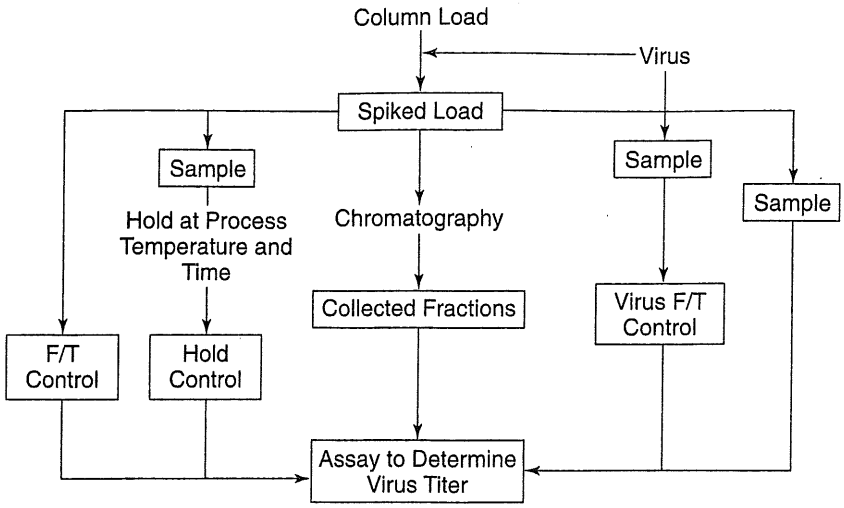

Figure 3. Clearance of virus by a column chromatography system.

Study design: Figure 3 shows the general approach to study design for assessing viral clearance by chromatography. The column load is the material applied to the chromatography column. The load material in small-scale viral clearance studies should be comparable to material from clinical or commercial manufacturing in terms of composition and physicochemical attributes. The virus stock is a freshly thawed virus material of predetermined infectivity titer that is used to perform virus spiking of the column load material and also is used in the interference study. The virus stock can be sampled to determine the actual virus titer immediately after thaw. The spiked load control and additional samples should be tested immediately or immediately frozen. The titer of the frozen sample and other samples will be determined at the end of the chromatography step run. In certain cases, filtration of the spiked load with a 0.22-µm or 0.45-µm prefilter may be necessary to remove any viral aggregates formed when the process intermediate was spiked with the model virus.

The chromatography step experiment for viral clearance should closely mimic the actual manufacturing process step parameters. It should be performed under worst-case conditions (e.g., flow, protein concentration, and elution conditions) if they have been established. The product pool or multiple fractions can be collected, and the virus titer can be determined in each collected fraction (such as flow-through, washes, pre-peak, peak, post-peak, strip, and others). The titers of virus in all the fractions that comprise the product pool are used to calculate the clearance factor of the step. Interference and cytotoxicity studies also can be performed on collected fractions, if warranted. For the duration of the step, the hold control (the load plus virus) should be kept at the temperature at which the chromatography is performed. At the end of the chromatography run, the hold control should be titrated along with other collected samples/fractions (these can be titrated immediately, or if not feasible, can be frozen and tested at a later time). Samples are evaluated using virus titration assays. Test samples may require neutralization to appropriate pH levels for cell-culture-based titration assays.

GLOSSARY

(See also *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩, *Glossary*.)[1]

Process Robustness of Viral Clearance: Ability of the downstream process or a single-unit operation to exhibit the characteristic performance and effectiveness in clearing viruses after minor or moderate changes of standard operating parameters or conditions.

Process Solutions: Production intermediates that are obtained for viral clearance studies. They should be obtained from a full-scale production facility or from a scaled-down process that has been shown to be representative of full-scale production.

Production Process: The manufacturing processes for biotechnology products are often varied and complex, and they are typically divided into upstream and downstream processing. Upstream activities produce the protein of interest, usually by cell culture or fermentation, and considerations include integrity and quality of the process, cell banks, expression systems, cultivation, media, process/product purity, impurities, and contaminants. Downstream processing involves separation and purification of the bulk bioproduct to make it suitable for its end use, e.g., purification, sterilization, and final formulation. Downstream activities include filtration, centrifugation, precipitation, numerous chromatographic separations, sterilization by terminal filtration, or lyophilization.

Purification Process: Separating and isolating the product of interest, in its desired form, from the fermentation supernatant or cell homogenate.

Viral Clearance Effectiveness: The efficacy of the entire purification process or an individual process step within a purification process to clear viruses, as determined by the viral reduction factor. In general, for a virus clearance step to be considered effective 4 logs or more of clearance must be demonstrated with 95% confidence ($\alpha = 0.05$).

Viral Load: The amount of virus added to the load material of a purification step and then subjected to an inactivation/removal treatment. The experimental determination of viral load before and after treatment enables calculation of a reduction factor that is specific to the virus and the treatment used.

[1] Cf. glossary in ⟨1050⟩.

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Viral Reduction Factor (VRF): The VRF of an individual clearance step represents the $\log_{10}$ of the ratio of the virus load in the pretreatment material (used to challenge the clearance step) to the virus load in the post-treatment material. The overall VRF is calculated by adding all the individual steps with a $\log_{10}$ reduction factor >1. The $\log_{10}$ viral reduction factor is determined by the following equation:

$$Log_{10}initial\ virus\ load - log_{10}final\ virus\ load = Log_{10}Viral\ Reduction\ Factor$$

The $\log_{10}$ viral reduction value expresses levels of decreased viral contamination by factors of 10 that could be easily converted to percent reduction. For instance, a 1-log reduction is equivalent to a 90% reduction, a 2-log reduction is a 99% reduction, a 3-log reduction is a 99.9% reduction, and a 4-log reduction is a 99.99% reduction.

Virus Removal/Inactivation Validation: Virus removal/inactivation validation studies evaluate the capacity of the process to eliminate and/or to inactivate the viruses. Typically, this involves spiking the product with a known virus and then subjecting the product to inactivation/removal processes.

⟨1051⟩ CLEANING GLASS APPARATUS

Success in conducting many Pharmacopeial assays and tests depends upon the cleanliness of the glassware apparatus used. Usage of commercial detergents or inorganic reagents for cleaning should be used when necessary.

In all cases, it is important to verify that the cleaning procedure is appropriate for the particular test or assay being undertaken. This can be accomplished in a number of ways, including use of experimental controls or verification of cleaning by utilization of residue/residual testing to ensure removal of any potential contaminants. A statement should be included in the cleaning protocol describing how the success of the cleaning procedure will be assessed.

For optical measurements, special care is required for cleaning containers, but the use of chromic acid or highly alkaline solutions should be avoided.

Some particular tests, though not inclusive, wherein the use of clean glassware is critical for success include the following: pyrogen and total organic carbon tests as well as assays of heparin sodium and vitamin B_{12} activity.

Selected references that might be helpful in obtaining additional information on cleaning glass apparatus are listed in the *Appendix*. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about the cleanliness of the glassware apparatus procedures mentioned in this chapter may also be found in most quantitative chemical analytical textbooks.

APPENDIX

Additional Information

Additional information and guidance can be found in the references listed below or in many quantitative chemical analytical textbooks:

1. Parenteral Drug Association. *Draft—Points to Consider for Cleaning Validation* (Technical Report Number 29). Bethesda, MD: Parenteral Drug Association; 1998.
2. Anderson NR. Container cleaning and sterilization. In: Olson WP, Groves MJ, eds. *Aseptic Pharmaceutical Manufacturing*. 1st ed. Buffalo Grove, IL: Interpharm Press; 1987:15–22.
3. Green C. Cleaning validation—application in the laboratory; Montalvo M. The cleaning validation policy and the cleaning validation plan; Verghese G, Kaiser N. Cleaning agents and cleaning chemistry; Verghese G, Lopolito P. Cleaning engineering and equipment design. In: Pluta PL, ed. *Cleaning and Cleaning Validation, Volume 1*. Bethesda, MD: Parenteral Drug Association; 2009.
4. Gordon AJ, Ford RA. Standard glassware cleaning solutions. In: Gordon AJ, Ford RA, eds. *The Chemist's Companion*. Hoboken, NJ: Wiley and Sons; 1973.

⟨1052⟩ BIOTECHNOLOGY-DERIVED ARTICLES—AMINO ACID ANALYSIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by amino acid analysis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. The footnote below is in the *USP* but is not in the *EP* or *JP*. Other characterization tests, also harmonized, are shown in *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties

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of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available* for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and is used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often, the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability

* *Suitable standards may be obtained from NIST (Gaithersburg, MD), Beckman Instruments (Fullerton, CA), Sigma Chemical (St. Louis, MO), Pierce (Rockford, IL), or Agilent (Palo Alto, CA).*

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of the amino acid analysis include sample preparation, high background spectral interference due to the quality of reagents and/or to laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reverse-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge;(2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids from the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this particular point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 N hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC-grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500° for 4 hours may be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine, the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., *Methods 4–11*) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but it requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of 1 minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. [NOTE—During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.]

- METHOD 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol.

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Procedure

Liquid Phase Hydrolysis: Place the protein or peptide sample in a hydrolysis tube, and dry. [NOTE—The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200 µL of *Hydrolysis Solution* per 500 µg of lyophilized protein. Freeze the sample tube in a dry ice–acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110° for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis: This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution*. The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

• METHOD 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution: 2.5 M MESA solution.

Vapor Phase Hydrolysis: About 1 to 100 µg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to between 170° to 185° for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

• METHOD 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution: a solution containing 7 M hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis: About 10 to 50 µg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166° for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

• METHOD 4

Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation Solution: The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubating at room temperature for 1 hour.

Procedure: The protein/peptide sample is dissolved in 20 µL of formic acid, and heated at 50° for 5 minutes; then 100 µL of the *Oxidation Solution* is added. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The oxidation is allowed to proceed for 10 to 30 minutes. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

• METHOD 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis: The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis Solution*. This technique allows better tyrosine recovery than *Method 4*, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

• METHOD 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis: The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution*. As an approach to limit variability and to compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine per mol protein. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

• METHOD 7

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

Reducing Solution: Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container, and mix.

Procedure: Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 µm of mercury or 6.7 Pa), and incubate at about 100° for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine per mol protein to improve accuracy in the pyridylethyl-cysteine recovery. Longer incubation times for the

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pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

• METHOD 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions: Prepare and filter three solutions: 1 M Tris hydrochloride (pH 8.5) containing 4 mM edetate disodium (*Stock Solution 1*), 8 M guanidine hydrochloride (*Stock Solution 2*), and 10% of 2-mercaptoethanol in water (*Stock Solution 3*).

Reducing Solution: Prepare a mixture of *Stock Solution 2* and *Stock Solution 1* (3:1) to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M Tris hydrochloride.

Procedure: Dissolve about 10 µg of the test sample in 50 µL of the *Reducing Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

• METHOD 9

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock Solutions: Prepare as directed for *Method 8*.

Carboxymethylation Solution: Prepare a solution containing 100 mg of iodoacetamide per mL of alcohol.

Buffer Solution: Use the *Reducing Solution*, prepared as directed for *Method 8*.

Procedure: Dissolve the test sample in 50 µL of the *Buffer Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the *Carboxymethylation Solution* in a 1.5 fold ratio per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [NOTE—If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethylcysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

• METHOD 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [NOTE—The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution: a solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 M sodium hydroxide.

Procedure: Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the *Reducing Solution*. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

• METHOD 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by Asx, while glutamine and glutamic acid residues are added and represented by Glx. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions: Prepare and filter three solutions: a solution of 10 mM trifluoroacetic acid (*Solution 1*), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (*Solution 2*), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per mL (*Solution 3*).

Procedure: In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of *Solution 1* or *Solution 2* and 2 mL of *Solution 3*. Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α-, β-diaminopropionic and α-, γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid assayed contents with underivatized and BTI-derivatized acid hydrolysis. [NOTE—The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues.]

METHODOLOGIES OF AMINO ACID ANALYSIS GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenyl-methylchloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reverse-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives

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of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, and chromatographic systems. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

• **Method 1—Postcolumn Ninhydrin Detection General Principle**

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acids, give a purple color, and show maximum absorption at 570 nm. The imino acids, such as proline, give a yellow color, and show maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

• **Method 2—Postcolumn OPA Fluorometric Detection General Principle**

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method I*. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this method exist.

Although OPA does not react with secondary amines (imino acids, such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound, such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes of pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limit is considered to be a few tens of pmol level for most of the amino acid derivatives. Response linearity is obtained in the range of a few pmol level to a few tens of nmol level. To obtain good compositional data, a sample greater than 500 ng before hydrolysis is best suited for the amino acid analysis of protein/peptide.

• **Method 3—Precolumn PITC Derivatization General Principle**

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reverse-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, a sample larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino analysis of proteins/peptides.

• **Method 4—Precolumn AQC Derivatization General Principle**

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reverse-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reverse-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reverse-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in the concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent byproduct, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ($t_{1/2}$< 15 seconds) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to be ranging from about 40 fmol to 320 fmol for each amino acid, except for Cys. The detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 µM to 200 µM with

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correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

- **Method 5—Precolumn OPA Derivatization General Principle**

Precolumn derivatization of amino acids with *o*-phthalaldehyde (OPA) followed by reverse-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol and 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reactions, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

Precolumn derivatization of amino acids with OPA is followed by reverse-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of the OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

- **Method 6—Precolumn DABS-Cl Derivatization General Principle**

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reverse-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acid derivatives, can be separated on an ODS column of a reverse-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyze the imino acids, such as proline, together with the amino acids, at the same degree of sensitivity. DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids, such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, or methanesulfonic acid, described for *Method 2* in *Protein Hydrolysis*. The other acid-labile residues, asparagine and glutamine, can also be analyzed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI, described for *Method 11* in *Protein Hydrolysis*.

The nonproteinogenic amino acid, norleucine, cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analyzed with reliability, and only 10 ng to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

- **Method 7—Precolumn FMOC-Cl Derivatization General Principle**

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reverse-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions, in aqueous solution, and is completed in 30 seconds. The derivatives are stable, with only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by reverse-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetic acid buffer, methanol, and acetonitrile (50:40:10) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives that are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 µM to 50 µM is obtained for most amino acids.

- **Method 8—Precolumn NBD-F Derivatization General Principle**

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reverse-phase HPLC separation with fluorometric detection is used.

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reverse-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives that are separated in 35 minutes. *E*-aminocaproic acid can be used as an internal standard because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as that for the precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive and might be advantageous for NBD-F against OPA.

The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 mg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine

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residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Calculations

Amino Acid Mole Percent: This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein/peptide under investigation is unknown. This information can be used to corroborate the identity of a protein and has other applications. Carefully identify and integrate the peaks obtained as directed for each *Procedure*. Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100r_U/r$$

in which r_U is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples: This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in µg, of each recovered amino acid by the formula:

$$mM_W/1000$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and M_W is the molecular weight, for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m/(1000M/M_{WT})$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in µg, of the protein; and M_{WT} is the molecular weight of the unknown protein.

Known Protein Samples: This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine), and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically, a greater than 5% variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m/m_S$$

in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and m_S is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

▲APPENDIX

Amino Acid Analysis Procedures

The examples of the specific procedures for each *Method* described in *Methodologies of Amino Acid Analysis* are shown.

• METHOD 1—POSTCOLUMN NINHYDRIN DETECTION

One method for postcolumn ninhydrin detection is shown below. Many other methods are also available, with instruments and reagents available commercially.

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Mobile Phase Preparation

Solution A: Transfer about 1.7 g of anhydrous sodium citrate and 1.5 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 3.0.

Solution B: Transfer about 1.7 g of anhydrous sodium citrate and 0.7 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 4.3.

Solution C: Prepare a solution containing 5% of sodium chloride, 1.9% of anhydrous sodium citrate, and 0.1% of phenol in water, and adjust to a pH of 6.

Column Regeneration Solution: Prepare a solution containing 0.8% of sodium hydroxide in water, and adjust to a pH of 13.

Mobile Phase: Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

Postcolumn Reagent: Transfer about 18 g of ninhydrin and 0.7 g of hydrindantin to 900 mL of a solution containing 76.7% of dimethyl sulfoxide, 0.7% of dihydrate lithium acetate, and 0.1% of acetic acid, and mix for at least 3 hours under inert gas, such as nitrogen. [NOTE—This reagent is stable for 30 days if kept between 2° and 8° under inert gas.]

Buffer Solution: Prepare a solution containing 2% of anhydrous sodium citrate, 1% of hydrochloric acid, 0.5% of thiodiglycol, and 0.1% of benzoic acid in water, and adjust to a pH of 2.

Chromatographic System: The liquid chromatograph is equipped with a detector with appropriate interference filters at 440, 570, or 690 nm and a 4.0-mm × 120-mm column that contains 7.5-µm sulfonated styrene-divinylbenzene copolymer packing. The flow rate is about 14 mL per hour. The system is programmed as follows. Initially equilibrate the column with *Solution A*; at 25 minutes, the composition of the *Mobile Phase* is changed to 100% *Solution B*; and at 37 minutes, the composition is changed to 100% *Solution C*. At 75 minutes into the run, the last amino acid has been eluted from the column, and the column is regenerated with *Column Regeneration Solution* for 1 minute. The column is then equilibrated with *Solution A* for 11 minutes before the next injection. The column temperature is programmed as follows. The initial temperature is 48°; after 11.5 minutes, the temperature is increased to 65° at a rate of 3° per minute; at about 35 minutes, the temperature is increased to 77° at a rate of 3° per minute; and finally at about 52 minutes, the temperature is decreased to 48° at a rate of 3° per minute.

Procedure and Postcolumn Reaction: Reconstitute the lyophilized protein/peptide hydrolysate in the *Buffer Solution*, inject an appropriate amount into the chromatograph, and proceed as directed for *Chromatographic System*. As the amino acids are eluted from the column, they are mixed with the *Postcolumn Reagent*, which is delivered at a flow rate of 7 mL per hour, through a tee. After mixing, the column effluent and the *Postcolumn Reagent* pass through a tubular reactor at a temperature of 135°, where a characteristic purple or yellow color is developed. From the reactor, the liquid passes through a colorimeter with a 12-mm flow-through cuvette. The light emerging from the cuvette is split into three beams for analysis by the detector with interference filters at 440, 570, or 690 nm. The 690-nm signal may be electronically subtracted from the other signals for improved signal-to-noise ratios. The 440-nm (imino acids) and the 570-nm (amino acids) signals may be added in order to simplify data handling.

• **METHOD 2—POSTCOLUMN OPA FLUOROMETRIC DETECTION**

One method of postcolumn OPA fluorometric detection is shown below.

Mobile Phase Preparation

Solution A: Prepare a solution of sodium hydroxide, citric acid, and alcohol in HPLC-grade water having a 0.2 N sodium concentration and containing 7% of alcohol (w/v), adjusted to a pH of 3.2.

Solution B: Prepare a solution of sodium hydroxide and citric acid in HPLC-grade water having a 0.6 N sodium concentration, adjusted to a pH of 10.0.

Solution C: 0.2 N sodium hydroxide.

Mobile Phase: Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

Postcolumn Reagent Preparation

Alkaline Buffer: Prepare a solution containing 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, and adjust to a pH of 10.0.

Hypochlorite Reagent: To 1 L of *Alkaline Buffer*, add 0.4 mL of sodium hypochlorite solution (10% chlorine concentration). [NOTE—The hypochlorite solution is stable for 2 weeks.]

OPA Reagent: Transfer 2 g of *N*-acetyl-L-cysteine and 1.6 g of OPA to a 15-mL volumetric flask, dissolve in and dilute with alcohol to volume, and mix. Transfer this solution and 4 mL of 10% aqueous polyethylene (23) lauryl ether to a 1-L volumetric flask, dilute with 980 mL of *Alkaline Buffer*, and mix.

Chromatographic System: The liquid chromatograph is equipped with a fluorometric detector set to an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.0-mm × 150-mm column that contains 7.5-µm packing L17. The flow rate is about 0.3 mL per minute, and the column temperature is set at 50°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 20 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B*; then there is a step change to 40% *Solution A* and 60% *Solution B*; over the next 18 minutes, the composition is changed linearly to 100% *Solution B* and held for 7 minutes; then there is a step change to 100% *Solution C*, and this is held for 6 minutes; then there is a step change to *Solution A*, and this composition is maintained for the next 8 minutes.

Procedure and Postcolumn Reaction: Inject about 1.0 nmol of each amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. As the effluent leaves the column, it is mixed with the *Hypochlorite Reagent*. The mixture passes through the first postcolumn reactor which consists of stainless steel 0.5-mm × 2-m tubing. A second postcolumn reactor of similar design is placed immediately downstream from the first postcolumn reactor and is used for the OPA postcolumn reaction. The flow rates for both the *Hypochlorite Reagent* and the *OPA Reagent* are 0.2 mL per minute, resulting in a total flow rate (i.e., *Hypochlorite Reagent*, *OPA Reagent*, and column effluent) of 0.7 mL per minute exiting from the postcolumn reactors. Postcolumn reactions are conducted at 55°. This results in a residence time of about 33 seconds in the OPA postcolumn reactor. After postcolumn derivatization, the column effluent passes through the fluorometric detector.

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• **METHOD 3—PRECOLUMN PITC DERIVATIZATION**

One method of precolumn PITC derivatization is described below.

Mobile Phase Preparation

Solution A: 0.05 M ammonium acetate, adjusted with phosphoric acid to a pH of 6.8.

Solution B: Prepare 0.1 M ammonium acetate, adjust with phosphoric acid to a pH of 6.8, and then prepare a mixture of this solution and acetonitrile (1:1).

Solution C: a mixture of acetonitrile and water (70:30).

Mobile Phase: Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

Derivatization Reagent Preparation

Coupling Buffer: a mixture of acetonitrile, pyridine, triethylamine, and water (10:5:2:3).

Sample Solvent: a mixture of water and acetonitrile (7:2).

Sample Derivatization Procedure: Dissolve the lyophilized test sample in 100 µL of the *Coupling Buffer*, and then dry in a vacuum centrifuge to remove any hydrochloride if a protein hydrolysis step was used. Dissolve the test sample in 100 µL of *Coupling Buffer*, add 5 µL of PITC, and incubate at room temperature for 5 minutes. The test sample is again dried in a vacuum centrifuge, and is dissolved in 250 µL of *Sample Solvent*.

Chromatographic System: The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 250-mm column that contains 5-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 52°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 15 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B*; over the next 15 minutes, the composition is changed linearly to 50% *Solution A* and 50% *Solution B*; then there is a step change to 100% *Solution C*, and this is held for 10 minutes; then there is a step change to 100% *Solution A*, and the column is allowed to equilibrate before the next injection.

Procedure: Inject about 1.0 nmol of each PITC-amino acid under test (10-µL sample in *Sample Solvent*) into the chromatograph, and proceed as directed for *Chromatographic System*.

• **METHOD 4—PRECOLUMN AQC DERIVATIZATION**

One method of precolumn AQC derivatization is shown below.

Mobile Phase Preparation

Solution A: Prepare a solution having a composition of 140 mM sodium acetate and 17 mM triethylamine, and adjust with phosphoric acid to a pH of 5.02.

Solution B: a mixture of acetonitrile and water (60:40).

Mobile Phase: Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Sample Derivatization Procedure: Dissolve about 2 µg of the test sample in 20 µL of 15 mM hydrochloric acid, and dilute with 0.2 M borate buffer (pH 8.8) to 80 µL. The derivatization is initiated by the addition of 20 µL of 10 mM AQC in acetonitrile, and allowed to proceed for 10 minutes at room temperature.

Chromatographic System: The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm and a 3.9-mm × 150-mm column that contains 4-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 0.5 minute, the composition of the *Mobile Phase* is changed linearly to 98% *Solution A* and 2% *Solution B*; then over the next 14.5 minutes to 93% *Solution A* and 7% *Solution B*; then over the next 4 minutes to 87% *Solution A* and 13% *Solution B*; over the next 14 minutes to 68% *Solution A* and 32% *Solution B*; then there is a step change to 100% *Solution B* for a 5-minute wash; over the next 10 minutes, there is a step change to 100% *Solution A*; and the column is allowed to equilibrate before the next injection.

Procedure: Inject about 0.05 nmol of each AQC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

• **METHOD 5—PRECOLUMN OPA DERIVATIZATION**

One method of precolumn OPA derivatization is shown below.

Mobile Phase Preparation

Solution A: a mixture of 100 mM sodium acetate (pH 7.2), methanol, and tetrahydrofuran (900:95:5).

Solution B: methanol.

Mobile Phase: Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent: Dissolve 50 mg of OPA in 1.25 mL of methanol (protein sequencing grade). Add 50 µL of 2-mercaptoethanol and 11.2 mL of 0.4 M sodium borate (pH 9.5), and mix. [NOTE—This reagent is stable for 1 week.]

Sample Derivatization Procedure: Transfer about 5 µL of the test sample to an appropriate container, add 5 µL of the *Derivatization Reagent*, and mix. After 1 minute, add not less than 20 µL of 0.1 M sodium acetate (pH 7.0). Use 20 µL of this solution for analysis. [NOTE—Use of an internal standard (e.g., norleucine) is recommended for quantitative analysis because of potential reagent volume variations in the sample derivatization. The sample derivatization is performed in an automated on-line fashion. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization.]

Chromatographic System: The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.6-mm × 75-mm column that contains 3-µm packing L3. The flow rate is about 1.7 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with 92% *Solution A* and 8% *Solution B*; over the next 2 minutes, the composition of the *Mobile Phase* is changed to 83% *Solution A* and 17% *Solution B*, and held for an additional 3 minutes; then changed to 54% *Solution A* and 46% *Solution B* over the next 5 minutes, and held for an additional 2 minutes; then changed to 34% *Solution A* and 66% *Solution B* over the next 2 minutes, and held for 1 minute; then over the next 0.3 minute changed to 20% *Solution A* and 80% *Solution B*, and held for an additional 2.6 minutes; and then finally over 0.6 minute changed to 92% *Solution A* and 8% *Solution B*, and held for an additional 0.6 minute.

Procedure: Inject about 0.02 nmol of each OPA-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

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• **Method 6—Precolumn DABS-Cl Derivatization**

One method for precolumn DABS-Cl derivatization is shown below.

Mobile Phase Preparation

Solution A: 25 mM sodium acetate (pH 6.5) containing 4% of dimethylformamide.

Solution B: acetonitrile.

Mobile Phase: Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent Preparation

Sample Buffer: 50 mM sodium bicarbonate, adjusted to a pH of 8.1.

Derivatization Reagent: Dissolve 1.3 mg of DABS-Cl in 1 mL of acetonitrile. [Note—This reagent is prepared fresh shortly before the derivatization step.]

Sample Dilution Buffer: Prepare a mixture of 50 mM sodium phosphate (pH 7.0) and alcohol (1:1).

Sample Derivatization Procedure: Dissolve the test sample in 20 µL of *Sample Buffer*, add 40 µL of *Derivatization Reagent*, and mix. The sample container is sealed with a silicon-rubber stopper, and heated to 70° for 10 minutes. During the sample heating, the mixture will become completely soluble. After the derivatization, dilute the test sample with an appropriate quantity of the *Sample Dilution Buffer*.

Chromatographic System: The liquid chromatograph is equipped with a 436-nm detector and a 4.6-mm × 250-mm column that contains packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 85% *Solution A* and 15% *Solution B*; over the next 20 minutes, the composition of the *Mobile Phase* is changed to 60% *Solution A* and 40% *Solution B*; over the next 12 minutes, the composition is changed to 30% *Solution A* and 70% *Solution B*, and held for an additional 2 minutes.

Procedure: Inject about 0.05 nmol of the DABS-amino acids into the chromatograph, and proceed as directed for *Chromatographic System*.

• **Method 7—Precolumn FMOC-Cl Derivatization**

One method for precolumn FMOC-Cl derivatization is shown below.

Mobile Phase Preparation

Acetic Acid Buffer: Transfer 3 mL of glacial acetic acid and 1 mL of triethylamine to a 1-L volumetric flask, and dilute with HPLC-grade water to volume. Adjust with sodium hydroxide to a pH of 4.20.

Solution A: a mixture of *Acetic Acid Buffer*, methanol, and acetonitrile (50:40:10).

Solution B: a mixture of acetonitrile and *Acetic Acid Buffer* (50:50).

Mobile Phase: Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent Preparation

Borate Buffer: Prepare a 1 M boric acid solution, and adjust with sodium hydroxide to a pH of 6.2.

FMOC-Cl Reagent: Dissolve 155 mg of 9-fluorenylmethyl chloroformate in 40 mL of acetone, and mix.

Sample Derivatization Procedure: To 0.4 mL of the test sample add 0.1 mL of *Borate Buffer* and 0.5 mL of *FMOC-Cl Reagent*. After about 40 seconds, extract the mixture with 2 mL of pentane, and then extract again with fresh pentane. The aqueous solution with amino acid derivatives is then ready for injection.

Chromatographic System: The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm and a 4.6-mm × 125-mm column that contains 3-µm packing L1. The flow rate is about 1.3 mL per minute. The system is programmed as follows. The column is equilibrated with *Solution A*, and this composition is maintained for 3 minutes; over the next 9 minutes, it is changed to 100% *Solution B*; then over the next 0.5 minute, the flow rate is increased to 2 mL per minute, and held until the final FMOC-amino acid is eluted from the column. The total run time is about 20 minutes.

Procedure: Inject not less than 0.01 nmol of each FMOC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. The FMOC-histidine derivative will generally give a lower response than the other derivatives.

• **Method 8—Precolumn NBD-F Derivatization**

One method for precolumn NBD-F derivatization is shown below.

Mobile Phase Preparation

Solution A: a solution of 10 mM sodium citrate containing 75 mM sodium perchlorate, adjusted with hydrochloric acid to a pH of 6.2.

Solution B: a mixture of acetonitrile and water (50:50).

Derivatization Reagent Preparation

Sample Buffer: a 0.1 M boric acid solution, adjusted with sodium hydroxide to a pH of 9.2.

Derivatization Reagent: Dissolve 5 mg of NBD-F in 1.0 mL of alcohol, and mix.

Sample Derivatization Procedure: Dissolve the test sample in 20 µL of *Sample buffer*, add 10 µL of *Derivatization Reagent*, and mix. The sample container is heated at 60° for 5 minutes. After the derivatization, dilute the test sample with 300 µL of *Solution A*.

Chromatographic System: The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm and a 4.6-mm × 150-mm column that contains 5-µm particle size ODS silica packing. The flow rate is about 1.0 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 94% *Solution A* and 6% *Solution B*; over the next 16 minutes, the composition is changed linearly to 63% *Solution A* and 37% *Solution B*; over the next 5 minutes, the composition is changed linearly to 62% *Solution A* and 38% *Solution B*; over the next 9 minutes, the composition is changed linearly to 100% *Solution B*, and held for an additional 5 minutes; then finally over 2 minutes, the composition is changed linearly to 94% *Solution A* and 6% *Solution B*; and then the column is allowed to equilibrate before the next injection.

Procedure: Inject about 15 pmol of each NBD-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

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⟨1053⟩ CAPILLARY ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by capillary electrophoresis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

INTRODUCTION

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution under the influence of a direct-current electric field. In this section we are describing four capillary electrophoresis methods: *Capillary Zone Electrophoresis, Capillary Gel Electrophoresis, Capillary Isoelectric Focusing,* and *Micellar Electrokinetic Chromatography.*

GENERAL PRINCIPLES

The migration velocity of the analyte under an electric field of intensity E is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electrical charge, molecular size, and shape) and the characteristics of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity, and additives). The electrophoretic velocity (v_{ep}) of a solute, assuming a spherical shape, is as follows:

$$v_{ep} = \mu_{ep} E = \left(\frac{q}{6\pi\eta r}\right)\left(\frac{V}{L}\right)$$

in which q is the effective charge of the solute; η is the viscosity of the electrolyte solution; r is the Stoke's radius of the solute; V is the applied voltage; and L is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent, called electroosmotic flow, is generated inside the capillary. Its velocity depends on the electroosmotic mobility (μ_{eo}), which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} E = \left(\frac{\varepsilon\zeta}{\eta}\right)\left(\frac{V}{L}\right)$$

in which ε is the dielectric constant of the buffer; ζ is the zeta potential of the capillary surface; and the other terms are as defined above.

The velocity of the solute (v) is given by the equation:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electroosmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run. The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is as follows:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l(L)}{V(\mu_{ep} + \mu_{eo})}$$

in which the other terms are as defined above.

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electroosmotic flow is from anode to cathode. The electroosmotic flow must remain constant from run to run to obtain good reproducibility in the migration velocity of the solutes. For some applications, it might be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition, and/or the pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions, the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case, the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

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$$N=\frac{(\mu_{ep}+\mu_{eo})(Vl)}{2DL}$$

in which D is the molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unleveled buffer reservoirs, can also significantly contribute to band dispersion. Separation between two bands (expressed by the resolution, R_S) can be obtained by modification of the electrophoretic mobility of the analytes, by the electroosmotic mobility induced in the capillary, and by increasing the efficiency for the band of each analyte as follows:

$$R_S=\frac{\sqrt{N}(\mu_{epb}-\mu_{epa})}{4(\bar{\mu}_{ep}+\mu_{eo})}$$

in which μ_{epa} and μ_{epb} are the electrophoretic mobilities of the two analytes to be separated; $\bar{\mu}_{ep}$ is the average electrophoretic mobility of the two analytes calculated as:

$$\bar{\mu}_{ep}=\tfrac{1}{2}(\mu_{epb}+\mu_{epa})$$

APPARATUS

An apparatus for capillary electrophoresis is composed of a high voltage controllable direct current power supply; two buffer reservoirs held at the same level and containing specified anodic and cathodic solutions; two electrode assemblies (cathode and anode) immersed in the buffer reservoirs and connected to the power supply; a separation capillary usually made of fused-silica, sometimes with an optical viewing window aligned with the detector, depending on the detector type, with the ends of the capillary placed in the buffer reservoirs and the capillary being filled with a solution specified in a given monograph; a suitable injection system; a detector capable of monitoring the amount of substance of interest passing through a segment of the separation capillary at a given time, generally based on absorption spectrophotometry (UV and visible), fluorimetry, conductimetric, amperometric, or mass spectrometric detection, depending on the specific applications, or even indirect detection to detect non-UV-absorbing and nonfluorescent compounds; a thermostatic system capable of maintaining a constant temperature inside the capillary, recommended to obtain good separation reproducibility; a recorder; and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum, or electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

It is expected that the capillary, the buffer solutions, the preconditioning method, the sample solution, and the migration conditions will be specified in the individual monograph. The electrolytic solution employed is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. To achieve reproducible migration time of the solutes, it would be necessary to develop, for each analytical method, a rigorous rinsing routine.

CAPILLARY ZONE ELECTROPHORESIS

Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. In this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow on the capillary (see *General Principles*). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

This mode of capillary electrophoresis is appropriate for the analysis of small (MW < 2000) and large (2000 < MW < 100,000) molecules. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of the separations are instrumental and electrolytic solution parameters.

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Instrumental Parameters

VOLTAGE

A Joule heating plot is useful in optimizing the applied voltage and column temperature. The separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result, viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

POLARITY

Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

TEMPERATURE

The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

CAPILLARY

The length and internal diameter of the capillary affects the analysis time, the efficiency of separations, and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which increases migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic, and anionic polymers are commercially available.

Electrolytic Solution Parameters

BUFFER TYPE AND CONCENTRATIONS

Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

To minimize band distortion, it is important to match buffer–ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH decreases electroosmotic flow and solute velocity.

BUFFER pH

The pH of the buffer can affect separation by modifying the charge of the analyte or additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point to below the isoelectric point changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

ORGANIC SOLVENTS

Organic modifiers, such as methanol, acetonitrile, and others, may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

ADDITIVES FOR CHIRAL SEPARATIONS

To separate optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides, or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutylether, etc.) moities. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account because it will influence the selectivity. The resolution of chiral separations is also controlled by the concentration of the chiral

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selector, the composition and pH of the buffer, and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution of separation.

CAPILLARY GEL ELECTROPHORESIS

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size because smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

Characteristics of Gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels are prepared inside the capillary by polymerization of monomers. One example of such a gel is a cross-linked polyacrylamide. This type of gel is usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. For protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate, and the sample is denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When nonreducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Optimization of separation in a cross-linked gel is obtained by modifying the separation buffer (see *Capillary Zone Electrophoresis*) and by controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the ratio of the cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of this type of gel, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers (i.e., linear polyacrylamide, cellulose derivatives, dextran, etc.) which can be dissolved in aqueous separation buffers, giving rise to a separation medium that also acts as a molecular sieve. These polymeric separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary with no electroosmotic flow. Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the dynamically coated gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A decrease in gel porosity leads to a decrease in the mobility of the solute for the same buffer. Both hydrodynamic and electrokinetic injection techniques can be used because the dissolution of these polymers in the buffer gives low viscosity solutions.

CAPILLARY ISOELECTRIC FOCUSING

Principle

In isoelectric focusing the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (polyaminocarboxylic acids), dissolved in the separation buffer.

The three basic steps in capillary isoelectric focusing are loading, focusing, and mobilization.

LOADING

Two methods may be employed.

Loading in One Step—The sample is mixed with ampholytes and introduced into the capillary by pressure or vacuum.

Sequential Loading—A leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone, and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough so as not to modify the pH gradient.

FOCUSING

When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charge, creating the pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point, and the current drops to very low values.

MOBILIZATION

If mobilization is required for detection, use one of the following methods. Three methods are available.

Method 1—Mobilization is accomplished during *Focusing*, under the influence of the electroosmotic flow when this flow is small enough to allow the focusing of the components.

Method 2—Mobilization is accomplished by application of positive pressure after *Focusing*.

Method 3—Mobilization is achieved after *Focusing*, by adding salts to the cathode reservoir or the anode reservoir, depending on the direction chosen for mobilization, in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir, which contains added salts, and pass the detector.

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The separation achieved is expressed as ΔpI and depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E), and the variation of the electrophoretic mobility of the analyte with the pH (−dμ/dpH):

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

Optimization

The major parameters that need to be considered in the development of separations are the following:

VOLTAGE

The use of high fields from 300 V/cm to 1,000 V/cm during *Focusing*.

CAPILLARY

Depending on the *Mobilization* strategy selected (see above), the electroosmotic flow must be reduced or suppressed. Coated capillaries tend to reduce the electroosmotic flow.

SOLUTIONS

The anode buffer reservoir is filled with a solution of a lower pH than the pI of the most acidic ampholyte, and the cathode reservoir is filled with a solution with a higher pH than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, like methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes covering many pH ranges are available and may also be mixed to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point (pI), whereas narrower ranges are employed to improve accuracy. Calibration can be made by correlating migration time with the isoelectric point of a series of standard protein markers. During *Focusing*, precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea, or zwitterionic buffers. However, depending on the concentration, urea can denature proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

Principle

Separation takes place in an electrolytic solution that contains a surfactant at a concentration above the critical micellar concentration (cmc). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed by the micelles according to the solute's partition coefficient. The technique can be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes maintaining the efficiency, speed, and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant, sodium dodecyl sulfate, although other surfactants, such as cationic surfactant cetyl trimethyl ammonium salts, have also been used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is used as surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, because the analyte can partition between the micelle and the aqueous buffer and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electroosmotic flow marker and that of the micelle; and the time elapsed between these two peaks is called the separation window. For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer and on the electrophoretic mobility of the solute in the absence of micelles.

Because the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k′), also referred to as mass distribution ratio (D_m), which is the ratio between the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k′ is as follows:

$$k' = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} = K\left(\frac{V_s}{V_M}\right)$$

in which t_r is the migration time of the solute; t_0 is the analysis time of the unretained solute obtained by injecting an electroosmotic flow marker that does not enter the micelle (e.g., methanol); t_{mc} is the micelle migration time measured by

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injecting a micelle marker, such as Sudan III, which migrates continuously associated in the micelle; K is the partition coefficient of the solute; V_S is the volume of the micellar phase; and V_M is the volume of the mobile phase.

The resolution between two closely-migrating solutes (R_S) is as follows:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b'}{k_b' + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k_a' \times \left(\frac{t_0}{t_{mc}}\right)}$$

in which N is the number of theoretical plates for one of the solutes; α is the selectivity; and k_a' and k_b' are retention factors for both solutes, respectively ($k_b' > k_a'$).

Similar, but not identical, equations give k′ and R_S values for electrically charged solutes.

Optimization

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

INSTRUMENTAL PARAMETERS

Voltage—Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross section of the capillary. This effect can be significant with high conductivity buffers, such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature—Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration, and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary—As in *Capillary Zone Electrophoresis,* length and internal diameter of the capillary contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electrical fields, working at constant voltage, and will increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation, for a given buffer and electrical field, and consequently broadening of the sample band.

ELECTROLYTIC SOLUTION PARAMETERS

Surfactant Type and Concentration—The type of surfactant, as the stationary phase in chromatography, affects the resolution because it modifies separation selectively. The log k′ of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. When k′ approaches the value of

$$\sqrt{t_{mc}/t_0}$$

resolution in MEKC reaches a maximum. Modifying the concentration of surfactant in the mobile phase changes the resolution.

Buffer pH—pH does not modify the partition coefficient of nonionized solutes, but it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and, therefore, increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic Solvents—To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers generally decreases migration time and selectivity of the separation. The addition of organic modifiers affects critical micellar concentration, thus, a given surfactant concentration can be used only with a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, the absence of the partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible, because in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for Chiral Separations—For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts, *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions that contain micellized achiral surfactants.

Other Additives—Selectivity can be modified by adding chemicals to the buffer. Addition of several types of cyclodextrins to the buffer is also used to reduce the interaction of hydrophobic solutes with the micelle, increasing the selectivity for this type of compound. The addition of substances able to modify solute-micelle interactions by adsorption on the latter has been used to improve the selectivity of the separations in MEKC. These additives may consist of a second surfactant (ionic or nonionic), which gives rise to mixed micelles or metallic cations that dissolve in the micelle and form coordination complexes with the solutes.

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Quantification

Peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response. Dividing the peak areas by migration time will also compensate for the different responses of sample constituents with different migration times. Where an internal standard is used, check that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of a component or components being determined. When indicated, the percentage of one (or more) components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all the peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. The parameters include the following: retention factor k′ used only for *Micellar Electrokinetic Chromatography,* apparent number of theoretical plates (N), the symmetry factor (A_S), and the resolution (R_S). Note that in previous sections, the theoretical expressions for N and R_S have been described, but more practical equations that allow for the determination of these suitability parameters using the electropherograms are described below.

Apparent Number of Theoretical Plates

The apparent number of theoretical plates (N) may be calculated from the formula:

$$N = 5.54\ (t_R/w_h)^2$$

in which t_R is the migration time or distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak corresponding to the component; and w_h is the peak width at half-height.

Resolution

The resolution (R_S) between peaks of similar heights of two components may be calculated from the formula:

$$R_S = 1.18\ (\ t_{R2} - t_{R1}\ )/(w_{h1} + w_{h2})$$

$$t_{R2} > t_{R1}$$

in which t_{R1} and t_{R2} are the migration times or distances along the baseline between the point of injection and the perpendiculars dropped from the maxima of two adjacent peaks; and w_{h1} and w_{h2} are the peak widths at half-height.

When appropriate, the resolution (R_S) may also be calculated by measuring the height of the valley (H_v) between two partly resolved peaks in a standard preparation, the height of the smaller peak (H_p), and calculating the peak-to-valley ratio:

$$p/v = H_p/H_v$$

Symmetry Factor

The symmetry factor of a peak (A_S) may be calculated using the formula:

$$A_s = w_{0.05}/2d$$

in which $w_{0.05}$ is the width of the peak at one-twentieth of the peak height; and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Other suitability parameters include tests for area repeatability (standard deviation of areas or of area/migration time) and tests for migration time repeatability (standard deviation of migration time). Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use a migration time relative to an internal standard.

Signal-to-Noise Ratio

A test for the verification of the signal-to-noise ratio for a standard preparation or the determination of the limit of quantification may also be useful for the determination of related substances. The detection limit and quantification limit correspond to a signal-to-noise ratio of 3 and 10, respectively. The signal-to-noise ratio (S/N) is calculated as follows:

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$$S/N = 2H/h$$

in which H is the height of the peak corresponding to the component concerned in the electropherogram obtained with the specified reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height; and h is the range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

⟨1054⟩ BIOTECHNOLOGY-DERIVED ARTICLES—ISOELECTRIC FOCUSING

This chapter provides guidance and procedures used for the characterization of biotechnology-derived articles by isoelectric focusing. This chapter is harmonized with the corresponding chapters in *JP* and *EP*. Other characterization tests, also harmonized, are shown in the USP general information chapters *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Biotechnology-Derived Articles—Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric points. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electrical field, the ampholytes migrate in the gel to create a pH gradient. In some cases, gels containing an immobilized pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralized and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentration effect is called "focusing". Increasing the applied voltage or reducing the sample load results in improved separation of bands. The applied voltage is limited by the heat generated because the heat must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel while allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate two neighboring bands, as follows:

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

in which D is the diffusion coefficient of the protein; dpH/dx is the pH gradient; E is the intensity of the electric field, in volts per centimeter; and $-d\mu/dpH$ is the variation of the solute mobility with the pH in the region close to the pI. Because D and $-d\mu/dpH$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Better resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pI values differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes, whereas immobilized pH gradients can resolve protein differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

From an operational point, special attention must be paid to sample characteristics and/or preparation. Salt in a sample can be problematic, and it is best to prepare the sample, if possible, in deionized water or 2% ampholytes using dialysis or gel filtration if necessary. The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a colored protein (e.g., hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some procedures the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins; the IEF gel can be used as a limit test when the density of a band on IEF is compared

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subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

APPARATUS

An apparatus for isoelectric focusing consists of a controllable generator for constant potential, current, and power. Potentials of 2500 V have been used and are considered optimal under a given set of operating conditions. Supply of up to 30 W of constant power is recommended. The apparatus also includes a rigid plastic isoelectric focusing chamber that contains a cooled plate of suitable material to support the gel; and a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length, and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

Preparation of the Gels

MOLD

The mold is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D), and clamps to hold the structure together (see *Figure 1*).

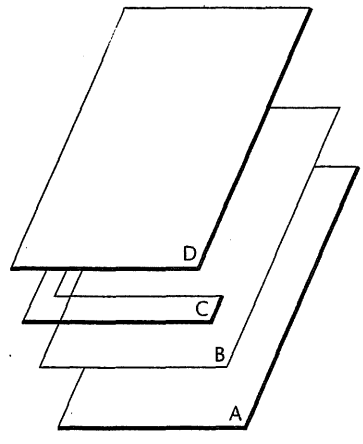

Figure 1. Mold

7.5% POLYACRYLAMIDE GEL

Dissolve 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the individual monograph, and dilute to 10 volumes with water. Mix carefully, and degas the solution.

PREPARATION OF THE MOLD

Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate, and fit the clamps. Before use, place the mixture on a magnetic stirrer, and add 0.25 volumes of a 100 g/L solution of ammonium persulfate and 0.25 volumes of tetramethylenediamine. Immediately fill the space between the glass plates of the mold with the solution.

FIXING SOLUTION FOR ISOELECTRIC FOCUSING POLYACRYLAMIDE GEL

Mix 35 g of sulfosalicylic acid and 100 g of trichloroacetic acid in 1000 mL of water.

COOMASSIE STAINING SOLUTION AND DESTAINING SOLUTION

Use the same solutions indicated in general information chapter *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.

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Procedure

Dismantle the *Mold*, and using the polyester film, transfer the gel onto the cooled support wetted with a few mL of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the individual monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel, and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some procedures, the gel has precast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut two strips of paper to the length of the gel, and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the individual monograph. Apply these paper wicks to each side of the gel several mm from the edge. Fit the cover so that the electrodes are in contact with the wicks (with respect to the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the individual monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forceps, remove the sample application strips and the two electrode wicks. Immerse the gel in *Fixing Solution for Isoelectric Focusing Polyacrylamide Gel*. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution, and add 200 mL of *Destaining Solution*. Incubate with shaking for 1 hour. Drain the gel, and add *Coomassie Staining Solution*. Incubate for 30 minutes. Destain the gel by passive diffusion with *Destaining Solution* until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram, as prescribed in the individual monograph.

Variations to the Detailed Procedure (Subject to Validation)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These variations include the use of commercially available precast gels and of commercial staining and destaining kits; the use of immobilized pH gradients; the use of rod gels, and the use of cassettes of different dimensions, including ultra-thin (0.2 mm) gels; variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper; the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability; the inclusion of a prefocusing step; the use of automated instrumentation; and the use of agarose gels.

VALIDATION OF ISOELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed, they must be validated. The following criteria may be used to validate the separation: formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points; comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined; and any other validation criteria as prescribed in the individual monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in individual monographs. Variations may include the addition of urea in the gel (a 3 M concentration is often satisfactory to keep the protein in solution, but up to 8 M can be used). Some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein. Other variations include the use of alternative staining methods and the use of gel additives such as nonionic detergents (e.g., octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO) and the addition of ampholyte to the sample to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

1. Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments, samples should not be applied close to either electrode. During method development, the analyst can try applying the protein in three positions on the gel (e.g., middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.
2. A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilized pH gradients may be used to address this problem.
3. Efficient cooling (approximately 4°) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

⟨1055⟩ BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by peptide mapping. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not

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harmonized with the other two pharmacopeias are marked by the symbol ♦. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

INTRODUCTION

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the formation of peptide fragments, followed by separation and identification of the resultant fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a Reference Standard or Reference Material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product or to detect the presence of protein variant. •The validation scheme presented differentiates between qualification of the method at an early stage in the regulatory process, the Investigational New Drug (IND) level, and full validation in support of New Drug Application (NDA), Product License Application (PLA), or Marketing Authorization Application (MAA). The validation concepts described are consistent with the general information chapter *Validation of Compendial Procedures* ⟨1225⟩ and with the International Conference on Harmonization (ICH) document *Analytical Methods Validation*.♦

THE PEPTIDE MAP

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a Reference Standard or Reference Material. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in *Table 1*. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	C-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin, EC 3.4.23.1 and EC 3.4.23.2	Nonspecific digest
	Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50	C-terminal side of Lys
	Glutamyl endopeptidase; (from *S. aureus* strain V8), EC 3.4.21.19	C-terminal side of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Asp-N endoproteinase), EC 3.4.24.33	*N*-terminal side of Asp
	Clostripain, EC 3.4.22.8	C-terminal side of Arg

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Table 1. Examples of Cleavage Agents *(continued)*

Type	Agent	Specificity
Chemical	Cyanogen bromide	*C*-terminal side of Met
	2-Nitro-5-thiocyanobenzoic acid	*N*-terminal side of Cys
	o-Iodosobenzoic acid	*C*-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

Pretreatment of Sample

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the Cleavage Agent

Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the Protein

Under certain conditions, it might be necessary to concentrate the sample, or to separate the protein from added substances and stabilizers used in the formulation of the product if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map as a result of side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of Optimal Digestion Conditions

Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH—The digestion mixture pH is empirically determined to ensure the optimal performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

Temperature—A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4° because at higher temperatures it will precipitate during digestion.

Time—If a sufficient amount of sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid that does not interfere with the tryptic map, or by freezing.

Amount of Cleavage Agent—Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein-to-protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents except the test protein.

Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for the separation of peptides are shown in *Table 2*.

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Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography—High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

In this section, a most widely used reverse-phase HPLC (RP-HPLC) method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration are also recommended.

Chromatographic Column

The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size and silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica particles 3 to 10 µm in diameter (L7) and of octadecylsilane chemically bonded to porous silica or ceramic microparticles 3 to 10 µm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles 5 to 10 µm in diameter (L26) packing.

Solvent

The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% of trifluoroacetic acid is added. If necessary, add isopropyl alcohol or *n*-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile Phase

Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, because shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile-containing trifluoroacetic acid is also used quite often.

Gradient Selection

Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become "marker" peaks for the test.

Isocratic Selection

Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

Other Parameters

Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or as versatile as UV detection.

System Suitability

The section *System Suitability* under *Chromatography* ⟨621⟩ provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is the comparison with a Reference

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Standard or Reference Material, which is treated exactly as the article under test. The use of a USP Reference Standard in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram should be included with the USP Reference Standard or Reference Material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection, and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant proteins is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the USP Reference Standard or Reference Material for the specified protein. The use of a digested USP Reference Standard or Reference Material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a Reference Standard can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the USP Reference Standard or Reference Material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and USP Reference Standard or Reference Material digest. If all peaks in the sample digest and in the USP Reference Standard or Reference Material digest have the same relative retention times and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and USP Reference Standard or Reference Material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches, for example the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides, have been proposed. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between USP Reference Standard or Reference Material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and is likely to introduce error into the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the USP Reference Standard or Reference Material. The possibility of autohydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when *N*-terminal sequencing and amino acid analysis are used, the analytical separation is scaled up. Because scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to ensure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase digestion and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF analyzers as well as fast atom bombardment

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(FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

•THE USE OF PEPTIDE MAPPING FOR GENETIC STABILITY EVALUATION

A validated peptide map can be used to assess the integrity of the predicted primary sequence of a protein product (i.e., its genetic stability). It can also be used to determine lot-to-lot consistency of the biotechnology-derived product process. Furthermore, the performance of the protein expression of the production system is best assessed by peptide mapping of the expressed protein. Peptide maps of protein produced at various times of the protein expression process, including a point well beyond the normal protein expression time, compared with those of a USP Reference Standard or Reference Material, will evaluate the genetic stability of the expression system as a function of time.

Variant protein sequences can arise from a genetic variation at the DNA level (point mutation) or as an error in the translation process. A validated peptide map is the best approach to the detection of protein variants. However, the limitations of the peptide mapping itself must be taken into consideration. The detection of a structured variant is possible only if the corresponding peptide variant is easily isolated and characterized. To establish genetic stability will require the use of a battery of biochemical methods, provided that the variants have properties different from those of the "normal" protein.•

•VALIDATION

Critical Factors

Validation of peptide mapping requires that a protocol be designed, outlining in detail the experiment to be conducted and the criteria for acceptance of the map. Criteria for acceptance of mapping include detection limit, specificity, linearity, range, accuracy, precision, and reagent stability. Reproducibility of the peptide map is a critical element in the utilization of such a map as an identity test and for confirming genetic stability. Those technical aspects of peptide mapping that influence the reproducibility of the map will be discussed.

The setting of limits, with respect to quantification (peak area or height) and identification (retention times) for the selected group of relevant peaks is based on empirical observations. These limits detect significant differences between the sample and USP Reference Standard or Reference Material within a series of analyses.

Another critical issue is the recovery of peptides and its impact on peak area determination and reproducibility and on the establishment of acceptance criteria. The recovery criteria address all aspects of test methodology, from digestion to chromatographic conditions. Determination of peptide recovery includes quantitative amino acid analysis, spike addition, radiolabeling, and UV summation. An overall recovery of about 80% is considered satisfactory. Recovery of individual peptides is more problematic and is handled on a case-by-case basis. The critical factors considered in the validation of a peptide map are as follows.

Written Test Procedures

These procedures include a detailed description of the analytical method in which reagents, equipment, sample preparation, method of analysis, and analysis of the data are defined.

Validation Protocol

A protocol is prepared that contains a procedure for test validation.

Acceptance Criteria

The criteria can be minimal at the early stages, but need to be better defined as validation studies progress.

Reporting of Results

Results from the validation study are documented with respect to the analytical parameters listed in the validation protocol.

Revalidation of the Test Procedure

If the method used requires alteration that could affect the analytical parameter previously assessed in the validation of the procedure, the test procedure must be revalidated. Significant changes in the processing of the article, in laboratories performing the analysis, in formulation of the bulk or the finished products, and in any other significant parameter will require revalidation of the methods.

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Requirements

PRECISION

Intratest Precision—This is a measure of the reproducibility of peptide mapping. The two critical steps in peptide mapping are fragmentation (i.e., digestion) and separation of peptides. An acceptable precision occurs where the absolute retention times and the relative peak areas are constant from run to run, and the average variation in retention time is small relative to that of a selected internal reference peak. The reproducibility of the map can be enhanced if a temperature-controlled column oven is used, if an extensive equilibration of the system is performed prior to the start of the test, if a blank (control digest mixture without protein) is run first to minimize "first run effects," and if a USP Reference Standard or a Reference Material digest is interspersed periodically with test samples to evaluate chromatographic drift.

The criteria for validation of the fragmentation step are similar to those described below for separation of peptides, but they are met for consecutive tests of a series of separately prepared digests of the protein under test.

The criteria for validation of the separation of peptides step include the following:

1. The average standard deviation of the absolute retention times of all major peaks for a set of consecutive tests of the same digest does not exceed a specified acceptance criterion.
2. The average standard deviation of absolute peak area for all fully resolved major peaks does not exceed a specified percentage.

Intertest Precision—This is a measure of the reproducibility of the peptide mapping when the test is performed on different days, by different analysts, in different laboratories, with reagents or enzymes from different suppliers or different lots from the same supplier, with different instruments, on columns of different makes or columns of the same make from different lots, and on individual columns of the same make from the same lot. Although it would be desirable, from a scientific perspective, to validate all of these variables in terms of their impacts on precision, a practical approach is to validate the test using those variables most likely to be encountered under operational conditions. Additional variables can be included when needed.

The experimental design allows the analyst to make comparisons using peak retention times and areas that are expressed relative to a highly reproducible internal reference peak within the same chromatogram. The relative peak area is expressed as the ratio of the peak area to that of the internal reference peak. The relative retention time can be expressed as the difference between the absolute retention time and that of the reference peak. The use of relative values eliminates the need to make separate corrections for differences due to injector-to-injector volumes, units of measure for peak areas, column dimensions, and instrument dead volumes. The variability in the retention times and peak areas for the *Intertest Precision* experiments is expected to be slightly higher than the variability observed for *Intratest Precision*.

ROBUSTNESS

Factors such as composition of the *Mobile Phase*, protease quality or chemical reagent purity, column variation and age, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

Mobile Phase—The composition of the *Mobile Phase* is optimized to obtain the maximum resolution of peptides throughout the elution profile. A balance between optimal resolution and overall reproducibility is desired. A lower pH might improve peak separation but might shorten the life of the column, resulting in lack of reproducibility. Peptide maps at a pH above and below the pH of the procedure are compared to the peptide map obtained at the pH of the procedure and checked for significant differences; they are also reviewed with respect to the acceptance criteria established in the validation protocol.

Protease Quality or Chemical Reagent Purity—A sample of the USP Reference Standard or Reference Material for the protein under test is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and carboxymethylation reagents.

Column Considerations—Column-to-column variability, even within a single lot, can affect the performance of the column in the development of peptide maps. Column size may also lead to significant differences. A USP Reference Standard or Reference Material of the protein under test is digested and the digest is chromatographed on different lots of column from a single manufacturer. The maps are then evaluated in terms of the overall elution profile, retention times, selectivity resolution, and recovery. To evaluate the overall lifetime of the column in terms of robustness, perform a peptide mapping test on different columns and vary significantly the number of injections (e.g., from 10 injections to 250 injections). The resulting maps can then be compared for significant differences in peak broadening, peak area, and overall resolution. As a column ages, an increase in back pressure might be observed that might affect the peptide maps. A sensible precaution in the use of peptide mapping columns is to select alternative columns in case the original columns become unavailable or are discontinued. Perform a peptide mapping test using equivalent columns from different manufacturers, and examine the maps. Differences in particle shape and size, pore size and volume, carbon load, and end-capping can lead to significant differences in retention times, elution profile selectivity, resolution, and recovery. Slight modifications in the gradient profile may be required to achieve equivalency of mapping when using columns from different manufacturers.

[NOTE—The equivalency between instrumentation used for the validation of the test and for routine quality control testing should be considered. It might be preferable to use the same HPLC system for all applications. Otherwise, equivalency of the systems is determined, which may require some changes in the chromatographic test conditions.]

Digest Stability—The length of time a digest can be kept before it is chromatographed, as well as the conditions under which the digest is stored before chromatography, is assessed. Several aliquots from a single digest are stored at different storage conditions and chromatographed. These maps are then evaluated for significant differences.

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REPRODUCIBILITY

Determination of various parameters indicated above is repeated using the same USP Reference Standard or Reference Material and test sample in at least two different laboratories by two analysts equipped with similar HPLC systems. The generated peptide maps are evaluated for significant differences.♦

⟨1056⟩ BIOTECHNOLOGY-DERIVED ARTICLES—POLYACRYLAMIDE GEL ELECTROPHORESIS

INTRODUCTION

Scope

Polyacrylamide gel electrophoresis is used for the qualitative characterization of proteins in biological preparations, for control of purity, and for quantitative determinations.

Purpose

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins. Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given in *Validation of the Test* (below).

CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibers and pores that is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerization usually is catalyzed by a free radical–generating system composed of ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel through manipulating the acrylamide concentration, analysts can optimize the resolution of the method for a given protein product. Thus, a given gel is physically characterized by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component of electrophoretic mobility. In the case of proteins, the electrophoretic mobility depends on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration, and the pH of the buffer; by the temperature and the field strength; and by the nature of the support material.

DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000–100,000 Da. It is possible to extend this mass range by various techniques (e.g., gradient gels and particular buffer system). For instance, tricine–sodium dodecyl sulfate (SDS) gels, using tricine instead of glycine (in the method described here) as the trailing ion in the electrophoresis running buffer, can separate very small proteins and peptides under 10,000–15,000 Da.

Denaturing polyacrylamide gel electrophoresis using glycine SDS (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and is the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged, and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS–polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent–polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes migrate toward the anode in a predictable manner; low-molecular-mass complexes migrate faster than larger ones. The molecular mass of a protein therefore can be estimated from its relative mobility in calibrated SDS-PAGE, and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, can change the apparent molecular mass of a protein, because SDS does not bind to a carbohydrate moiety in a manner similar to that of a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.

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Depending on the extent of glycosylation and other posttranslational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

Reducing Conditions

Polypeptide subunits and three-dimensional structure often are maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) results in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, analysts can reasonably calculate the molecular mass of the polypeptide by linear regression (or, more closely, by nonlinear regression) in the presence of suitable molecular mass standards.

Nonreducing Conditions

For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS–protein complexes migrate more slowly than their SDS–polypeptide subunits. In addition, nonreduced proteins may not be completely saturated with SDS and hence may not bind the detergent in a constant mass ratio. Moreover, intrachain disulfide bonds constrain the molecular shape, usually in such a way that reduces the Stokes radius of the molecule, thereby reducing the apparent molecular mass, M_r. This makes molecular mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides because it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS

The most popular electrophoretic method for the characterization of complex mixtures of proteins uses a discontinuous buffer system involving two contiguous but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large-volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution and drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed, with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS–protein complexes to form into a thin zone (stack) and to migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS–proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane (Tris) and glycine. Molecular sieving causes the SDS–polypeptide complexes to separate on the basis of their molecular masses.

PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

This section describes the preparation of gels using particular instrumentation. This does not apply to precast gels. For precast gels or any other commercially available equipment, the manufacturer's instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and when the purity of the reagents used is not sufficient, a pretreatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionized with a mixed-bed (anion–cation exchange) resin to remove acrylic acid and other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

Gel Stock Solutions

30% ACRYLAMIDE–BISACRYLAMIDE SOLUTION

Prepare a solution containing 290 g of acrylamide and 10 g of methylenebisacrylamide per liter of water. Filter.

AMMONIUM PERSULFATE SOLUTION

Prepare a small quantity of solution having a concentration of 100 g/L of ammonium persulfate. [NOTE—Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Because ammonium persulfate decomposes rapidly, fresh solutions must be prepared daily.]

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TEMED

Use an electrophoresis-grade reagent.

SDS SOLUTION

This is a 100 g/L solution of electrophoresis-grade SDS.

1.5 M BUFFER SOLUTION

Dissolve 90.8 g of Tris in 400 mL of water. Adjust the pH to 8.8 with hydrochloric acid, and dilute to 500.0 mL with water.

1 M BUFFER SOLUTION

Dissolve 60.6 g of Tris in 400 mL of water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 500.0 mL with water.

Assembling the Gel-Molding Cassette

Clean the two glass plates (size, e.g., 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers, and the silicone rubber tubing (e.g., 0.6 mm diameter × 35 cm length) with mild detergent; rinse extensively with water, followed by dehydrated alcohol; and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with nonsilicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer, and follow the long side of the glass plate. While holding the tubing with one finger along the long side, twist the tubing again and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment, and hold the mold together by hand pressure.

Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the gel mold, thus forming the bottom of the gel mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while the clamps were placed. The gel mold is now ready for pouring the gel.

Preparation of the Gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel, because the composition of the two gels in acrylamide–bisacrylamide, buffer, and pH are different.

PREPARATION OF THE RESOLVING GEL

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in *Table 1*. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in *Table 1*, swirl, and pour immediately into the gap between the two glass plates of the mold. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipet, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerization.

Table 1. Preparation of the Resolving Gel

	Component Volume (mL) per Gel Mold Volume Below							
Solution component	**5 mL**	**10 mL**	**15 mL**	**20 mL**	**25 mL**	**30 mL**	**40 mL**	**50 mL**
6% Acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% Acrylamide–Bisacrylamide Solution	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% Acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% Acrylamide–Bisacrylamide Solution	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3

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Table 1. Preparation of the Resolving Gel *(continued)*

	Component Volume (mL) per Gel Mold Volume Below							
Solution component	**5 mL**	**10 mL**	**15 mL**	**20 mL**	**25 mL**	**30 mL**	**40 mL**	**50 mL**
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% Acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% Acrylamide–Bisacrylamide Solution	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% Acrylamide–Bisacrylamide Solution	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
30% Acrylamide–Bisacrylamide Solution	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Buffer Solution	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% Acrylamide–Bisacrylamide Solution	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

PREPARATION OF THE STACKING GEL

After polymerization is complete (about 30 min), pour off the isobutanol, and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in *Table 2*. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter:

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0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in *Table 2*. Swirl, and pour immediately into the gap between the two glass plates of the mold directly onto the surface of the polymerized resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position, and allow it to polymerize at room temperature.

Table 2. Preparation of the Stacking Gel

	Component Volume (mL) per Gel Mold Volume Below							
Solution component	**1 mL**	**2 mL**	**3 mL**	**4 mL**	**5 mL**	**6 mL**	**8 mL**	**10 mL**
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% Acrylamide–Bisacrylamide Solution	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Buffer Solution	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
SDS Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
Ammonium Persulfate Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Preparation of the Sample

Unless otherwise specified in the specific monograph, the samples can be prepared as follows:

SDS-PAGE SAMPLE BUFFER (CONCENTRATED)

Dissolve 1.89 g of Tris, 5.0 g of sodium lauryl sulfate, and 50 mg of bromophenol blue in water. Add 25.0 mL of glycerol, and dilute to 100 mL with water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 125 mL with water.

SDS-PAGE SAMPLE BUFFER FOR REDUCING CONDITIONS (CONCENTRATED)

Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Add 25.0 mL of 2-ME. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Alternatively, DTT can be used as reducing agent instead of 2-ME. In this case prepare the sample buffer as follows: Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Immediately before use, add DTT to a final concentration of 100 mM.

SDS-PAGE RUNNING BUFFER

Dissolve 151.4 g of Tris, 721.0 g of glycine, and 50.0 g of sodium lauryl sulfate in water, and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with water, and mix. Measure the pH of the diluted solution. The pH is between 8.1 and 8.8.

SAMPLE SOLUTION (NONREDUCING CONDITIONS)

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer (Concentrated)*.

SAMPLE SOLUTION (REDUCING CONDITIONS)

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer for Reducing Conditions (Concentrated)* containing 2-ME (or DTT) as the reducing agent.

The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: Keep for 5 min in a boiling water bath or in a block heater set at 100°, and then chill. (Note that temperature and time may vary in the monograph because protein cleavage may occur during the heat treatment.)

MOUNTING THE GEL IN THE ELECTROPHORESIS APPARATUS AND ELECTROPHORETIC SEPARATION

After polymerization is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE Running Buffer* to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing, and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, because this will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse each well with *SDS-PAGE Running Buffer*. Prepare

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the test and reference solutions in the recommended sample buffer, and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells.

Start the electrophoresis using the conditions recommended by the equipment manufacturer. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness, and electrophoresis running time and current or voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is near the bottom of the gel stop the electrophoresis. Remove the gel assembly from the apparatus, and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel, and immediately proceed with staining.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS—GRADIENT CONCENTRATION GELS

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient-forming apparatus. Ready-to-use gradient gels are commercially available with specific recommended protocols.

Gradient gels offer some advantages over fixed-concentration gels. Some proteins that co-migrate on fixed-concentration gels can be resolved within gradient gels. During electrophoresis the proteins migrate until the pore size stops further progress, and therefore a stacking effect occurs, resulting in sharper bands. According to *Table 3*, gradient gels also allow separation of a wider range of protein molecular masses than do single, fixed-concentration gels.

Table 3 gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular ranges. Note that other gradient shapes (e.g., concave) can be prepared for specific applications.

Table 3. Acrylamide Gradient Percentages Recommended for Expected Protein Molecular Weights

Acrylamide (%)	Protein Range (kDa)
5–15	20–250
5–20	10–200
10–20	10–150
8–20	8–150

Gradient gels also are used for molecular mass determination and protein purity determination.

DETECTION OF PROTEINS IN GELS

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods, and commercial kits are available. For example, fluorescent stains are visualized using a fluorescent imager and often provide a linear response over a wide range of protein concentrations—often several orders of magnitude, depending on the protein.

Coomassie staining has a protein detection level of approximately 1–10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels, and a band containing 10–100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of one or two orders of magnitude has been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining, but the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e., when the analyst deems the staining satisfactory. Wide dynamic ranges of reference proteins are important to use because they help assess the intra-experimental sensitivity and linearity. All gel-staining steps are done while wearing gloves, at room temperature, with gentle shaking (e.g., on an orbital shaker platform), and using any convenient container.

Staining Reagents

DESTAINING SOLUTION

Prepare a mixture of 1 volume of glacial acetic acid, 4 volumes of methanol, and 5 volumes of water.

COOMASSIE STAINING SOLUTION

Prepare a 1.25 g/L solution of acid blue 83 in *Destaining Solution*. Filter.

FIXING SOLUTION

To 250 mL of methanol, add 0.27 mL of formaldehyde, and dilute to 500.0 mL with water.

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SILVER NITRATE REAGENT

To a mixture of 3 mL of concentrated ammonia and 40 mL of 1 M sodium hydroxide, add 8 mL of a 200 g/L solution of silver nitrate, dropwise, with stirring. Dilute to 200 mL with water.

DEVELOPER SOLUTION

Dilute 2.5 mL of a 20 g/L solution of citric acid and 0.27 mL of formaldehyde to 500.0 mL with water.

BLOCKING SOLUTION

A 10% (v/v) solution of acetic acid.

Coomassie Staining

Immerse the gel in a large excess of *Coomassie Staining Solution*, and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *Destaining Solution*. Change the *Destaining Solution* several times until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. More rapid destaining can be achieved by including a few grams of anion-exchange resin or a small sponge in the *Destaining Solution*. [NOTE—The acid–alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid, 4 volumes of methanol, and 5 volumes of water for 1 h before it is immersed in the *Coomassie Staining Solution*.]

Silver Staining

Immerse the gel in a large excess of *Fixing Solution*, and allow it to stand for 1 h. Remove the *Fixing Solution*, add fresh *Fixing Solution*, and incubate for at least 1 h or overnight, if convenient. Discard the *Fixing Solution*, and wash the gel in a large excess of water for 1 h. Soak the gel for 15 min in a 1% (v/v) solution of glutaraldehyde. Wash the gel twice for 15 min in a large excess of water. Soak the gel in fresh *Silver Nitrate Reagent* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of water. Immerse the gel for about 1 min in *Developer Solution* until satisfactory staining has been obtained. Stop the development by incubation in the *Blocking Solution* for 15 min. Rinse the gel with water.

RECORDING THE RESULTS

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, gel-scanning systems with data analysis software are commercially available to photograph and analyze the wet gel immediately.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of glycerol for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/L solution of glycerol.

Drying of stained SDS polyacrylamide gels is one of the methods to have permanent documentation. This method frequently results in gel cracking during drying between cellulose films.

Immerse two sheets of porous cellulose film in water, and incubate for 5–10 min.

Place one of the sheets on a drying frame. Carefully lift the gel, and place it on the cellulose film. Remove any trapped air bubbles, and pour a few mL of water around the edges of the gel. Place the second sheet on top, and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

MOLECULAR MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of prestained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and are loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, mark the position of the bromophenol blue tracking dye to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or R_F. Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_F values. Unknown molecular masses can be estimated by linear regression analysis (more accurately, by nonlinear regression analysis) or interpolation from the curves of log M_r against R_F if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

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VALIDATION OF THE TEST

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers, e.g., across 80% of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the R_F. If the plot has a sigmoidal shape, then only data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity also must be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve as a system suitability check of the experiment.

QUANTITATION OF IMPURITIES

SDS-PAGE is often used as a limit test for impurities. When impurities are quantitated by normalization to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein, as described in the introduction of the section *Detection of Proteins in Gels*, the linear range can vary but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentrations.

When the impurity limit is specified in the individual monograph, analysts should prepare a reference solution corresponding to that level of impurity by diluting the test solution. For example, when the limit is 5%, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities can be quantified by normalization to the main band, using an integrating densitometer, or by image analysis.

⟨1057⟩ BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY

Delete the following:

▲This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are provided in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.▲ (USP 1-Aug-2019)

Change to read:

INTRODUCTION

▲A number of factors needs to be considered when choosing a procedure for the measurement of total protein content in a pharmacopeial preparation. Those factors include the complexity of the sample, constraints that impact the procedure, the required accuracy/variability of the procedure, and the desired analyst contact or automation associated with the procedure (e.g., sample availability, analysis time). The choice of a total protein measurement procedure balances these and other factors to meet the application need.

An analytical separation technique (e.g., HPLC, capillary electrophoresis) may be the best option as a total protein measurement procedure when analyzing a complex sample. Separating the sample into various components and integrating relevant peaks on a chromatogram or electropherogram can be used to manage 1) sample components that interfere with spectrophotometric protein measurements; 2) a mixture of proteins or peptides in the sample, including excipients (e.g., human serum albumin), where the individual protein components need to be quantitated; 3) other sample attributes that are being assessed (e.g., identity, impurity quantitation) during the analysis; 4) a limited sample quantity available for analysis; or 5) the balance between analysis time, instrument complexity, and other factors that favor a separation procedure over a spectrophotometric procedure.

Amino acid quantitation may also be suitable for quantitation of total protein and is often used as a primary method of calibration for other total protein measurement procedures (e.g., determination of an extinction coefficient used with protein absorbance at 280 nm).

Spectrophotometric procedures for total protein measurement are often employed for the analysis of pharmacopeial preparations due to their simplicity, high sample throughput for a given analysis time, and low cost.▲ (USP 1-Aug-2019)

Many of the total protein assay methods described below can be performed successfully using kits from commercial sources.▲▲ (USP 1-Aug-2019)

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Add the following:

▲SAMPLE EXTRACTION METHODS FOR TOTAL PROTEIN ASSAYS

Accurate measurement of total protein cannot always be accomplished if there are interfering substances in the sample. This interference can be observed with spectrophotometric methods of total protein measurement. In order to accurately measure the total protein content in samples with these interfering substances, a method to remove these substances needs to be part of the procedure and the total procedure (extraction in combination with a measurement method) should be validated as suitable for its intended purpose.

It is possible that an interfering substance exhibits a known reproducible effect on the assay that can be eliminated by using an appropriate blank containing the interfering substance to calibrate the spectrophotometer to zero. As an alternative, a spike control added to the *Test solution* or sample matrix can be used to adjust the test result based on the spike recovery.

Sample extraction techniques often involve precipitating protein from the sample, leaving the interfering substances in the liquid phase that is subsequently removed, and then solubilizing the precipitated protein in the assay buffer for analysis. Precipitating protein from the sample can also have the advantage of concentrating proteins from a dilute solution, thereby allowing a sample that was too dilute to use with a particular spectrophotometric procedure to now be within the useful range of the method. Gel filtration procedures to remove an interfering substance can result in sample dilution which should also be considered when developing the method of analysis. With all techniques, volumes of solutions used must be measured quantitatively to allow accurate calculation of concentration and dilution factors that are used in the final calculation of protein concentration in the starting sample.

The use of an extractive procedure may introduce a sample preparation error and affect the precision of the protein concentration result. When an extraction procedure is performed on *Test solutions*, it should also be performed for *Standard solutions*, system suitability solutions, and the *Blank*. Samples should be homogeneous and free of particles. Examples of several possible sample extraction procedures, depending upon the nature of the interfering substance, are described below but others may be demonstrated as suitable for use too.

Procedure 1—Protein Precipitation with Acetone

1. Add acetone to the sample to bring the concentration of acetone to 85%–90%.
2. Let the sample stand for 1 h. If necessary for appropriate recovery, hold the sample at 4° overnight.
3. Centrifuge the sample at 14,000 × g or greater for 10 min. Discard the supernatant.
4. Dissolve the pellet in the appropriate buffer for analysis.

Procedure 2—Protein Precipitation with Trichloroacetic Acid

SODIUM DEOXYCHOLATE REAGENT

Prepare a solution of 1.5 g/L sodium deoxycholate in water.

TCA REAGENT

Prepare a solution of 720 g/L trichloroacetic acid (TCA) in water.

PROCEDURE

1. Add 0.1 mL of *Sodium deoxycholate reagent* to 1.0 mL of *Test solution*.
2. Mix using a vortex mixer, and then incubate for 10 min.
3. Add 0.1 mL of *TCA reagent*, and mix on a vortex mixer.
4. Centrifuge at a minimum of 6700 × g for 30 min.
5. Discard the supernatant.
6. Dissolve the protein pellet in 1.0 mL of the appropriate buffer for analysis.

Procedure 3—Solid Phase Device Extraction

BUFFER PREPARATION

Prepare the appropriate buffer or solvent mixture depending upon the solid phase extraction (SPE) device being used for fractionation.

PROCEDURE

1. Load the sample onto the SPE device.
2. Wash with a suitable buffer or solvent mixture.
3. Elute the protein with an appropriate buffer or solvent mixture.

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Procedure 4—Column Gel Filtration

BUFFER PREPARATION

Prepare the appropriate buffer for the test method.

PROCEDURE

The protein is purified using either gravity or a centrifugal procedure per the manufacturer's instructions.[1] ▲(USP 1-Aug-2019)

Change to read:

▲TOTAL PROTEIN MEASUREMENT PROCEDURES

Ideally, the protein in the *Standard solution* that is used to create the calibration curve should be the same protein as in the *Test solution*. This may not be practical and the use of a generic protein in the *Standard solution* may be substituted. Bovine serum albumin is often used because it is readily available and can be purchased at high purity. However, using a generic protein to create the standard curve may not be the ideal choice for all assays depending on the principle of the assay and the protein structure responsible for the response. Due to the potential variation in responses between different proteins that may be used to create the standard curve, the protein used for the standard curve should always be reported. In addition (unless otherwise specified in the individual monograph) prepare the Reference Standard or reference material for the protein under test in the same buffer used to prepare the *Test solution*.▲(USP 1-Aug-2019)

Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of *Method 1*. ▲▲(USP 1-Aug-2019)If the buffer used to ▲solubilize▲(USP 1-Aug-2019) the protein has a high absorbance relative to ▲▲(USP 1-Aug-2019) water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation.

▲To determine whether the protein sample is contaminated with nucleic acid, a 260/280 ratio is recommended if no other methods are used to quantitate the nucleic acids. *Table 1* can be used as a guide for protein purity.

Table 1. 260/280 Ratio for Estimating Nucleic Acid Contamination and Protein Purity of the Sample

Protein (%)	Nucleic Acid (%)	260/280 Ratio
100	0	0.57
95	5	1.06
90	10	1.32
70	30	1.73▲(USP 1-Aug-2019)

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2–2 mg/mL.

STANDARD SOLUTION

Unless otherwise specified in the individual monograph, prepare a solution of Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the *Test solution*.

PROCEDURE

Concomitantly determine the ▲absorbance values▲(USP 1-Aug-2019) of the *Standard solution* and *Test solution* in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), using the buffer as the *Blank*. ▲[NOTE—Keep the *Test solution*, *Standard solution*, and buffer at the same temperature during testing.]▲(USP 1-Aug-2019) To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

[1] Suitable commercial columns are available. For example, PD-10 columns (available from GE Healthcare) for samples ranging from 1.0–2.5 mL, NAP-10 columns for samples up to 1.0 mL, NAP-5 columns for samples up to 0.5 mL, or another suitable alternative.

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LIGHT SCATTERING

The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the ▲*Test solution*.▲ (USP 1-Aug-2019) If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250–300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light scattering, determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Test solution* at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light scattering at 280 nm. Subtract the absorbance due to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-µm porosity or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

CALCULATIONS

Calculate the concentration, C_U, of protein in the test specimen by the formula:

$$\text{Result} = C_S(A_U/A_S)$$

in which C_S is the concentration of the *Standard solution*; and A_U and A_S are the corrected ▲absorbance values▲ (USP 1-Aug-2019) of the *Test solution* and the *Standard solution*, respectively (see ⟨857⟩).

Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic–tungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. ▲Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.▲ (USP 1-Aug-2019)

The effect of interfering substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement. ▲Alternatively, procedures described in *Sample Extraction Methods for Total Protein Assays* could be used to remove interfering substances from the test sample.

The Lowry assay can be used for protein concentrations of 5–100 µg/mL. Wider ranges may be acceptable if the range of standard concentrations selected results in a linear curve.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations between 5 and 100 µg of protein per mL, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*. An appropriate buffer will produce a pH in the range of 10.0–10.5.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

REAGENTS AND SOLUTIONS

Copper sulfate reagent: Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.
SDS solution: Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.
Sodium hydroxide solution: Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.
Alkaline copper reagent: Prepare a mixture of *Copper sulfate reagent, SDS solution,* and *Sodium hydroxide solution* (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.
Diluted Folin-Ciocalteu's phenol reagent: Mix 10 mL of Folin-Ciocalteu's phenol TS with 50 mL of water. Store in an amber bottle at room temperature.

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PROCEDURE

To 1 mL of each *Standard solution*, the *Test solution*, and the *Blank*, add 1 mL of *Alkaline copper reagent*, and mix. Allow to stand at room temperature for 10 min. Add 0.5 mL of the *Diluted Folin-Ciocalteu's phenol reagent* to each solution, mix each tube immediately, ▲and hold at room temperature for about 30 min.

[NOTE—Color development reaches a maximum in 20–30 min during incubation at room temperature, after which there is a gradual loss of color.]

Determine the absorbance values of the *Standard solutions* and *Test solution* at 750 nm with a suitable spectrophotometer, using the solution from the *Blank* to set the instrument to zero (see ⟨857⟩).▲ (USP 1-Aug-2019)

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the ▲absorbance values▲ (USP 1-Aug-2019) of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test solution*, determine the concentration of protein in the *Test solution*.

▲▲ (USP 1-Aug-2019)

Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the Brilliant Blue G dye binds to protein. The Brilliant Blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins. ▲There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 100 µg–1 mg/mL of protein, the concentrations being evenly spaced.

TEST SOLUTION

▲Solubilize▲ (USP 1-Aug-2019) a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

COOMASSIE REAGENT

Dissolve 100 mg of Brilliant Blue G[2] in 50 mL of alcohol. [NOTE—Not all dyes have the same Brilliant Blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman #1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [NOTE—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

PROCEDURE

Add 5 mL of the *Coomassie reagent* to 100 µL of each *Standard solution*, the *Test solution*, and the *Blank*, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. ▲Incubate at room temperature for a suitable period of time, and then determine the absorbance values▲ (USP 1-Aug-2019) of the solutions from the *Standard solutions* and *Test solution* at 595 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument to zero. [NOTE—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same. ▲For consistent and accurate results, the absorbance values for all *Standard solutions*, *Blank*, and *Test solutions* should be determined within a suitable period of time to assure reproducible results of adding the *Coomassie reagent*.▲ (USP 1-Aug-2019)]

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.]▲Plot the absorbance values of the *Standard solutions* versus the protein

[2] Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, ▲Islandia,▲ (USP 1-Aug-2019) NY) is an acceptable grade.

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concentrations, and use linear regression to establish the standard curve. Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

Method 4

This method, commonly referred to as the bicinchoninic acid (BCA) assay, is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{1+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement. ▲If substances that will cause interference in the test are present, proceed as directed in *Sample Extraction Methods for Total Protein Assays*. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 10–1200 µg/mL of protein, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

REAGENTS

BCA reagent: Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.
Copper sulfate reagent: Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.
Copper–BCA reagent: Mix 1 mL of *Copper sulfate reagent* and 50 mL of *BCA reagent*.

PROCEDURE

Mix 0.1 mL of each *Standard solution*, the *Test solution*, and the *Blank* with 2 mL of the *Copper–BCA reagent*. Incubate the solutions at 37° for 30 min, note the time, and allow ▲the mixtures▲ (USP 1-Aug-2019) to ▲cool▲ (USP 1-Aug-2019) to room temperature. Within 60 min following the incubation time, determine the ▲absorbance values for▲ (USP 1-Aug-2019) the *Standard solutions* and the *Test solution*▲▲ (USP 1-Aug-2019) at 562 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument ▲calibration▲ (USP 1-Aug-2019) to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature. ▲▲ (USP 1-Aug-2019)

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.]▲Plot the absorbance values of the solutions from the *Standard solutions* versus the protein concentrations and determine the standard curve best fitting the plotted points. Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the ▲▲ (USP 1-Aug-2019) development of absorbance at 545 nm. ▲This test shows minimal difference between IgG and albumin samples at the same concentration. Addition of the sodium hydroxide and the *Biuret reagent* as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the *Biuret reagent* will give IgG samples a higher response than albumin samples. The trichloroacetic acid method (described in *Procedure 2* of *Sample Extraction Methods for Total Protein Assays*) used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg/mL.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

▲Prepare the▲ (USP 1-Aug-2019) Reference Standard or reference material for the protein under test in ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution. ▲▲ (USP 1-Aug-2019) Dilute portions of this solution with ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution

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▲▲ (USP 1-Aug-2019) to obtain NLT 3 *Standard solutions* having concentrations of 0.5–10 mg/mL, the concentrations being evenly spaced. ▲▲ (USP 1-Aug-2019)

TEST SOLUTION

Prepare a solution of the test protein in ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution ▲▲ (USP 1-Aug-2019) having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution. ▲▲ (USP 1-Aug-2019)

BIURET REAGENT

Dissolve about 3.46 g of cupric sulfate in 10 mL of hot water, and allow to cool (Solution 1). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of sodium carbonate in 80 mL of hot water, and allow to cool (Solution 2). Mix Solution 1 and Solution 2, and dilute with water to 200 mL. The *Biuret reagent* is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

PROCEDURE

To 1 volume of ▲▲ (USP 1-Aug-2019) the *Test solution* add an equal volume of ▲6%▲ (USP 1-Aug-2019) sodium hydroxide solution ▲▲ (USP 1-Aug-2019) and mix. Immediately add a volume of *Biuret reagent* equivalent to 0.4 of the volume of the *Test solution*, and mix. Allow to stand at a temperature between 15°–25° for NLT 15 min. Within 90 min after the addition of the *Biuret reagent*, determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Standard solutions* and the solution from the *Test solution* at 545 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to ▲calibrate▲ (USP 1-Aug-2019) the instrument to zero. [NOTE—Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

CALCULATIONS

▲Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear. Plot the absorbance values of the *Standard solutions* versus the protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the line.▲ (USP 1-Aug-2019) A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. ▲Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

Method 6

This fluorometric method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with OPA and must be avoided or removed. Ammonia at high concentrations will react with OPA as well. The fluorescence obtained when amine reacts with OPA can be unstable. The use of automated procedures to standardize ▲the analysis▲ (USP 1-Aug-2019) may improve the accuracy and precision of the test.

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲prepare▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 10–200 µg/mL of protein, the concentrations being evenly spaced.

TEST SOLUTION

▲Solubilize▲ (USP 1-Aug-2019) a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

REAGENTS

Borate buffer: Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.

Stock OPA reagent: Dissolve about 120 mg of OPA in 1.5 mL of methanol, add 100 mL of *Borate buffer*, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

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OPA reagent: To 5 mL of *Stock OPA reagent* add 15 µL of 2-mercaptoethanol. Prepare at least 30 min prior to use. This reagent is stable for 1 day.

PROCEDURE

Adjust each of the *Standard solutions* and the *Test solution* to a pH between 8▲.0▲ (USP 1-Aug-2019) and 10.5. Mix 10 µL of the *Test solution* and each of the *Standard solutions* with 100 µL of *OPA reagent*, and allow to stand at room temperature for 15 min. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see *Fluorescence Spectroscopy* ⟨853⟩), determine the fluorescent intensities of solutions from the *Standard solutions* and the *Test solution* at an excitation wavelength of 340 nm and an emission wavelength between 440–455 nm. [NOTE—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

CALCULATIONS

The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. ▲Determine the concentration of protein in the *Test solution* from the standard curve and the fluorescence of the *Test solution*.▲ (USP 1-Aug-2019)

Method 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method ▲since non-proteinaceous nitrogen will also be detected.▲ (USP 1-Aug-2019) Nitrogen analysis techniques destroy the protein under test and are not ▲stand-alone methods since they quantitate all nitrogen present. If either *Procedure 1* or *Procedure 2* below is used to determine protein content, then a suitable orthogonal method must also be used to verify that no other nitrogen-containing sources are present and contributing to the apparent total protein content.▲ (USP 1-Aug-2019)

PROCEDURE 1

Determine the nitrogen content of the protein under test as directed under *Nitrogen Determination* ⟨461⟩. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

PROCEDURE 2

Commercial instrumentation is available for nitrogen ▲(elemental)▲ (USP 1-Aug-2019) analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°), which produces nitric oxide (NO) and similar oxides of nitrogen (NO_x) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

CALCULATIONS

The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the Reference Standard or reference material.

⟨1058⟩ ANALYTICAL INSTRUMENT QUALIFICATION

INTRODUCTION

A large variety of analytical instruments, ranging from a simple apparatus to complex computerized systems, is used in the pharmaceutical industry to acquire data that will help ensure that products meet their specifications. Many of these instruments combine a metrological function with software control. There are many ways of demonstrating that an instrument is qualified and under control, and these can include qualification, calibration, validation, and maintenance. In order to ensure "fitness for purpose", an integrated approach, based upon a risk assessment, is recommended. For the purposes of this chapter, the term "instrument" includes any apparatus, equipment, instrument, or instrument system used in pharmacopeial analyses.

This informational chapter provides general guidance in a scientific, risk-based approach for carrying out an analytical instrument qualification (AIQ). Detailed instrument operating parameters to be qualified are found in the respective general chapters for specific instrument types. It is left to each laboratory to justify and document its specific approaches. The instrument owners/users and their management are responsible for assuring their instruments are suitably qualified.

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This chapter uses various terms, acronyms, and activities common to analytical laboratories and validation disciplines. These terms and activities may not be identical to their usages in all laboratories. The reader is encouraged to be flexible in interpreting the application of these terms and activities in the context and intent of this chapter.

The risk assessment for an AIQ enables the classification of the instrument to determine the extent of qualification and actions needed to demonstrate fitness for purpose. Generally, the more complex the instrument, or the higher the criticality of the measurement, the greater the amount of work that is required to ensure that quality data will be generated. In addition, attention must be paid to ensuring that data integrity and security are maintained.

Instruments can generally be classified as belonging to Groups A, B, or C. It should be noted that the same type of instrument can fit into one or more categories, depending on its intended use.

Group A includes the least complex, standard instruments that are used without measurement capability or user requirement for calibration, such as a magnetic stirrer or vortex mixer. Proper function is ensured by observation, and no further qualification activities are needed for this group.

Group B includes instruments that may provide a measurement or an experimental condition that can affect a measurement. Examples include a pH meter or an oven. Proper function of instruments in this group may require only routine calibration, maintenance, or performance checks. The extent of activities may depend on the criticality of the application. Generally, these instruments may have firmware but not software that is updated by the user.

Group C comprises analytical instruments with a significant degree of computerization and complexity, such as high-pressure liquid chromatographs and mass spectrometers. All elements of qualification, including software validation, must be considered to ensure proper functioning of instruments in this group.

COMPONENTS OF DATA QUALITY

There are four critical components involved in the generation of reliable and consistent data (quality data). *Figure 1* shows these components as layered activities within a quality triangle. Each layer adds to the overall quality. AIQ forms the base for generating quality data. The other components essential for generating quality data are analytical method validation, system suitability tests, and quality control check samples. These quality components are described below.

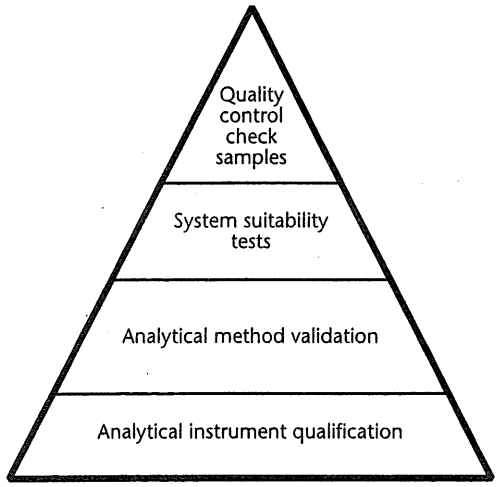

Figure 1. Components of data quality.

Analytical Instrument Qualification

AIQ is the collection of documented evidence that an instrument performs suitably for its intended purpose. Use of a qualified instrument in analyses contributes to confidence in the validity of generated data.

Analytical Method Validation

Analytical method validation is the collection of documented evidence that an analytical procedure is suitable for its intended use. Use of a validated procedure with qualified analytical instruments provides confidence that the procedure will generate test data of acceptable quality. Additional guidance on validation of compendial procedures may be found in *Validation of Compendial Procedures* ⟨1225⟩.

System Suitability Tests

System suitability tests verify that the system will perform in accordance with the criteria set forth in the procedure. These tests are performed along with the sample analyses to ensure that the system's performance is acceptable at the time of the test. *Chromatography* ⟨621⟩ presents a more detailed discussion of system suitability tests related to chromatographic systems.

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Quality Control Check Samples

Many analysts carry out their tests on instruments that have been standardized by using reference materials and/or calibration standards. Some analyses also require the inclusion of quality control check samples to provide an in-process or ongoing assurance of the test's suitable performance. In this manner, AIQ and analytical method validation contribute to the quality of analysis before analysts conduct the tests. System suitability tests and quality control checks help ensure the quality of analytical results immediately before or during sample analysis.

ANALYTICAL INSTRUMENT QUALIFICATION PROCESS

The following sections address the AIQ process in detail. The other three components of building quality into analytical data —analytical method validation, system suitability tests, and quality control check samples—are not within the scope of this chapter.

Qualification Phases

AIQ is not a single, continuous process but instead results from interconnected activities over the lifetime of the instrument. The first activity is the generation of a user requirements specification (URS), which defines the laboratory's particular needs and technical and operational requirements that are to be met. The subsequent qualification activities necessary to establish fitness for purpose may be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). PQ is also sometimes called user acceptance testing (UAT). This framework may be extended for complex systems to include functional specifications (FS) and factory acceptance testing (FAT), if appropriate. Some activities normally undertaken in IQ, OQ, or PQ may be satisfied during the instrument installation and start-up. This is sometimes referred to as site acceptance testing (SAT). It is more important that all required activities be performed in a logical order and scientifically sound manner than the exact allocation within the IQ/OQ/PQ framework. The activities may also be performed as an integrated framework.

Some AIQ activities cover more than one qualification phase, and analysts could potentially perform them during more than one of the phases. However, there is a need for a logical, specific order to the AIQ activities; for example, IQ activities such as configuration must occur before OQ can start. However, where appropriate, qualification activities and associated documentation may be combined together (e.g., IQ and OQ). All AIQ activities should be predefined and contemporaneously documented.

OQ tests are specifically designed to verify the instrument's correct functionality and operation according to specifications in the user's environment, as documented in the URS. Repeating all the testing at regular intervals may not be required. However, following preventative maintenance, critical operational parameters should be confirmed. Routine analytical tests do not constitute OQ testing.

When the instrument undergoes major repairs or modifications, this should be evaluated using change control. Relevant IQ, OQ, and/or PQ tests should be repeated to verify that the instrument continues to operate satisfactorily. If an instrument is moved to another location, an assessment should be made of what, if any, qualification stage should be repeated.

DESIGN QUALIFICATION

DQ is the documented collection of activities that define the functional and operational specifications and intended purpose of the instrument. DQ states what the laboratory wants the instrument to do and shows that the selected instrument is suitable. DQ may be performed by the instrument manufacturer or the user. It is expected that DQ requirements will be minimal for commercial, off-the-shelf instruments. Verification that the instrument specifications meet the desired functional requirements may suffice.

The supplier is generally responsible for robust design and maintaining documentation describing how the analytical instrument and any associated controlling software are manufactured (e.g., design specifications, functional requirements, and others) and tested, sometimes called factory acceptance tests. Nonetheless, the user should ensure that instruments are suitable for their intended application and may evaluate whether the supplier has adopted a quality system that provides for reliable instrumentation, software, and network connectivity. Users should also determine the supplier's capability to support installation, services, and training. This determination might be aided by the user's previous interaction with the supplier.

When use of an instrument changes or it is subject to a major upgrade, it may be necessary to review and/or update the user's DQ documentation.

INSTALLATION QUALIFICATION

IQ is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, is properly installed in the selected environment, and that this environment is suitable for the instrument. IQ applies to an instrument that is new or was pre-owned. For any instrument that exists on site but has not been previously qualified, or not qualified to current industry standards, existing documents should be collated and a risk assessment should be undertaken to determine the best course of action.

Relevant parts of IQ would also apply to a qualified instrument that has been transported to another location or is being reinstalled for other reasons, such as prolonged storage.

The activities and documentation typically associated with IQ are as follows.

Instrument delivery: Ensure that the instrument, software, manuals, supplies, and any other instrument accessories arrive as specified by the user and that they are undamaged. For a pre-owned or existing instrument, manuals and documentation should be obtained.

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Description: Document information about the instrument and all components, including supplier(s), model(s), serial number(s), software version(s), and location.
Utilities/Facility/Environment: Verify that the installation site satisfactorily meets supplier-specified environmental requirements.
Assembly and installation: Assemble and install the instrument, and perform any preliminary diagnostics and testing. Assembly and installation may be done by the supplier, service agents, specialized engineers, or qualified in-house personnel. Supplier-established installation tests provide a valuable baseline reference for determining instrument acceptance. Any abnormal event observed during assembly and installation merits documenting. IQ documentation packages purchased from a supplier should be reviewed to ensure that they are acceptable to the user before and after execution.
Software installation, network, and data storage: Some analytical systems require the installation of software onto a qualified computer and to be connected to a network for communications and data storage at the installation site. Information technology involvement is often required with computerized laboratory systems.
Installation verification: Perform the initial diagnostics and testing of the instrument after installation. When required, connect the instrument to the network, and check its functionality.

OPERATIONAL QUALIFICATION

OQ is the documented collection of activities necessary to demonstrate that an instrument will function according to its operational specification testing in the selected environment. OQ demonstrates fitness for the selected use, and should reflect URS. Testing activities in the OQ phase may consist of the following test parameters.
Fixed parameters: These tests measure the instrument's non-changing parameters such as length, height, weight, voltage inputs, acceptable pressures, and loads. If the manufacturer-supplied specifications for these parameters satisfy the user, the test requirements may be waived. However, if the user wants to confirm the parameters, testing can be performed at the user's site. Fixed parameters do not change over the life of the instrument, and therefore never need to be retested. [NOTE—These tests could also be performed during the IQ phase; if so, fixed parameters need not be retested as part of OQ testing.]
Software functions: Where applicable, OQ testing should include critical elements of the configured application software to show that the whole system works as intended. Functions to test would be those applicable to data capture, analysis of data, and reporting results under actual conditions of use as well as security, access control, and audit trail. The user can apply risk assessment methodologies and can leverage the supplier's software testing to focus the OQ testing effort.
Secure data storage, backup, and archiving: When applicable, test secure data handling, such as storage, backup, audit trails, and archiving at the user's site, according to written procedures.
Instrument function tests: Instrument functions required by the user should be tested to verify that the instrument operates as intended by the manufacturer. Supplier information is useful in identifying specifications for these parameters and in designing tests to verify that the instrument meets the supplier or user specifications in the user's environment.

The extent of OQ testing that an instrument undergoes depends on its intended applications, therefore no specific OQ tests for any instrument or application are offered in this chapter. Parameters to qualify are described in the general chapters pertaining to a specific analytical technique.

OQ tests can be modular or holistic. Modular testing of individual components of a system may facilitate interchanging of such components without requalification but requires a risk assessment to justify it. Holistic tests, which involve the entire system, demonstrate that the whole system complies with URS.

For OQ test packages purchased from a service provider or supplier, the user must review the material to assure themselves of the scientific soundness of the tests and compliance with applicable regulations. The user should review the documents before execution and approve the tests after execution to ensure completeness and accuracy of the completed document and the test data generated.
Software configuration and/or customization: Any configuration or customization of instrument software should occur before the OQ and be documented. Unless changes are needed for specific component tests, the OQ should be performed using the software configuration that will be used for routine analysis.

PERFORMANCE QUALIFICATION

PQ is the documented collection of activities necessary to demonstrate that an instrument consistently performs according to the specifications defined by the user, and is appropriate for the intended use. The PQ verifies the fitness for purpose of the instrument under actual conditions of use. After IQ and OQ have been performed, the instrument's continued suitability for its intended use is demonstrated through continued PQ.

The user must define the PQ plans, including test procedures, acceptance criteria, and frequency. Preventive maintenance plans and documentation of repairs and other changes are also a necessary part of the overall instrument qualification.

PQ may include the following activities.
Performance checks: A test or series of tests to verify the acceptable performance of the instrument for its intended use. PQ tests are usually based on the instrument's typical on-site applications and may consist of analyzing known components or standards. The tests should be based on good science and reflect the general intended use of the instrument. Some system suitability tests or quality control checks that are performed concurrently with the test samples can be used to demonstrate that the instrument is performing suitably. PQ tests may resemble those performed during OQ, but the specifications for PQ results may be set differently if required. Nevertheless, user specifications for PQ tests should demonstrate trouble-free instrument operation for the intended applications. As is the case with OQ testing, PQ tests may be modular or holistic.

Testing frequency depends on the ruggedness of the instrument and the criticality of the analytic method. Testing may be unscheduled; for example, each time the instrument is used. It may also be scheduled for regular intervals. Experience with the instrument can influence this decision, which should be documented. It may be useful to repeat the same PQ tests each time the instrument is used so that a history of the instrument's performance can be compiled. Alternatively, the instrument may be

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incorporated into an integrated support system to ensure that it remains continually qualified. System suitability tests that are performed concurrently with the test preparations may also ensure that the instrument is performing suitably.

Preventive maintenance and repairs: Periodic preventive maintenance activities are required for many instruments. This may include calibration. Document the preventive maintenance plans, including procedures and frequency as part of the AIQ package. When an instrument fails to meet PQ criteria or otherwise malfunctions, the cause of the failure must be investigated and documented. The instrument may require maintenance or repair. The relevant OQ or PQ test(s) should be repeated after the needed maintenance or repair to ensure that the instrument remains qualified.

Practices for PQ, change control, and periodic review: Each PQ, maintenance, and calibration activity should be documented. Change control should be established to control changes to the instrument configuration, including firmware and software. Critical instruments should have a periodic review to ensure that the system is still under control. Typical areas for review can include qualification/validation status, currency of user procedures, change control records, correctness and completeness of records produced by the system, backup and recovery of electronic records, and review and sign-off of test results.

The instrument owner/user and their management are responsible for this work, although portions can be carried out on his/her behalf by internal staff or external suppliers or service providers.

ROLES AND RESPONSIBILITIES

Users

Users are ultimately responsible for specifying their needs and ensuring that a selected instrument meets them, and that data quality and integrity are maintained. The user's group encompasses analysts, their supervisors, instrument specialists, and organization management. Users should be adequately trained in the instrument's use, and their training records should be maintained as required by the regulations.

Users should also be responsible for qualifying their instruments, because their training and expertise in the use of instruments make them the best-qualified group to design the instrument test(s) and specification(s) necessary for a successful AIQ. Consultants, instrument manufacturers or suppliers, validation specialists, and quality assurance personnel can advise and assist as needed, but the final responsibility for qualifying instruments and validating systems lies with the users, who must ensure that the instrument is maintained in a qualified state through routine performance of PQ.

Quality Unit

The role of the quality unit in AIQ remains the same as for any other regulated activity. Quality personnel are responsible for ensuring that the AIQ process meets compliance requirements, that processes are being followed, and that the intended use of the instrument is supported by complete, valid, and documented data.

Manufacturers, Suppliers, Service Agents, and Consultants

Manufacturers are responsible for designing and manufacturing the instrument, and ensuring the quality of relevant processes used in manufacturing and assembly of the instrument. Manufacturers should test the assembled instruments before shipping them to users. To aid the user, suppliers are responsible for developing meaningful specifications for the users to compare with their needs and aid selection.

Where used, software should be developed and tested using a defined life cycle and should have evidence of work performed to support major and minor revisions. Release notes should accompany each version of software released.

Finally, it is desirable that suppliers should notify all known users about hardware or software defects discovered after a product's release; offer user training, service, repair, and installation support; and invite user audits as necessary.

There should be a quality or technical agreement between the user organization and manufacturers, suppliers, service agents, or consultants who supply calibration, maintenance, qualification, or validation services; the agreement should define the scope of work and the responsibilities of the two parties.

SOFTWARE VALIDATION

There is an increasing inability to separate the hardware and software parts of modern analytical instruments. In many instances, the software is needed to qualify the instrument, and the instrument operation is essential when validating the software. Therefore, to avoid overlapping and potential duplication, software validation and instrument qualification can be integrated into a single activity.

Software used for analytical instruments can be classified into four groups: firmware, instrument control software, data acquisition software, and processing software. Although software validation is not the primary focus of this chapter, the following sections describe in which cases this activity is within the scope of the analytical instrument and system qualification.

One source of the validation of software is the guide *GAMP: A Risk-Based Approach to Compliant GxP Computerized Systems.*

Firmware

Computerized analytical instruments contain integrated chips with low-level software (firmware). Such instruments will not function without properly operating firmware, and in most cases users generally cannot alter firmware function. Firmware is

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therefore considered a component of the instrument itself. Indeed, the qualification of hardware is not possible without operating it via its firmware.

Thus, when the hardware (that is, the analytical instrument) is qualified at the user's site, the firmware is also essentially qualified. No separate on-site qualification of the firmware is needed. Whenever possible, the firmware version should be recorded as part of the IQ activities. Any changes made to firmware versions should be tracked through the change control of the instrument (see *Change Control*, below).

In some instruments, firmware can also be capable of fixed calculations on the acquired data. These calculations need to be verified by the user. Some instruments have firmware that enables users to define programs for the instrument's operation; similarly, these user-defined programs need to be defined and verified to demonstrate that they are fit for the intended purpose. Any user-defined programs should be placed under change control and, if possible, access should be restricted to authorized personnel.

Instrument Control, Data Acquisition, and Processing Software

Software for instrument control, data acquisition, and processing for many of today's computerized instruments is loaded on a computer connected to the instrument. Operation of the instrument is then controlled via the software, leaving fewer operating controls on the instrument. Also, the software is needed for data acquisition and post-acquisition calculations. Thus, both hardware and software, their functions inextricably intertwined, are critical to providing analytical results.

The software in this group can be classified into three types: 1) non-configurable software that cannot be modified to change the business process; 2) configurable software that includes tools from the supplier to modify the business process; and 3) configurable software with custom additions (i.e., custom software or macros to automate the business process).

The supplier of the system should develop and test the software according to a defined life cycle and provide users with a summary of the tests that were carried out. Ideally, this software development should be carried out under a quality management system.

At the user site, integrated qualification of the instrument, in conjunction with validation of the software, involves the entire system. This is more efficient than separating instrument qualification from validation of the software.

CHANGE CONTROL

Changes to qualified instruments, including software, become inevitable as suppliers add new features and correct known defects. However, implementing all such changes may not always benefit users. Users should therefore adopt changes they deem useful or necessary. Changes also occur due to repair, maintenance, or relocation of the instrument. A change control process should be in place to guide the assessment, execution, documentation, and approval of any changes to instrumentation.

Change control applies to all elements of qualification and may follow the general qualification process. Users should assess the effects of changes to determine what, if any, requalification activities are required. If implementation of the change is needed, install the changes to the system. Consider if the change will affect the ability of the instrument to meet the user requirements or if the user requirements have changed. Evaluate which of the existing OQ and PQ tests need revision, deletion, or addition as a result of the installed change.

After implementation, perform any required testing to evaluate the effects of the change. Document all details of the change. Include a description of the change and a rationale, and list appropriate identification (e.g., part and serial numbers of new components and versions of new software or firmware).

ANALYTICAL INSTRUMENT QUALIFICATION DOCUMENTATION

Documents obtained during qualification activities should be retained in an accessible manner. Where multiple instruments of one kind exist, documents common to all instruments and documents specific to an instrument may be stored separately. During change control, additional documents may supplement those obtained during the qualification process, and both sets of documents should be retained and maintained in a suitable manner that allows for appropriate protection and access.

GLOSSARY

[NOTE—The definitions of these terms may be different than in other *USP* general chapters.]

Calibration: An operation that, under specified conditions, in a first step, establishes a relation between the quantity values, with measurement uncertainties provided by measurement standards, and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication. Note that:

1. A calibration may be expressed by a statement, calibration function, calibration diagram, calibration curve, or calibration table. In some cases, it may consist of an additive or multiplicative correction of the indication with associated measurement uncertainty.
2. Calibration should not be confused with adjustment of a measuring system, often mistakenly called "self-calibration", or with verification of calibration.
3. Often, the first step alone in the above definition is perceived as being calibration.

Maintenance: Actions performed to keep an analytical instrument in a state of proper function so that it continues to operate within the boundaries set during qualification or validation.

Qualification: Action of proving that any instrument works correctly and delivers the expected results; demonstration of fitness for purpose.

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Software configuration: Adaptation of software functions to a business process using tools provided within the application by the supplier of the software.

Software customization: Changing the way software automates a business process by the addition of externally custom-coded software modules using a recognized programming language or the development of macros within the application software.

Software validation: Confirmation by examination and provision of objective evidence that software conforms to user needs and intended uses, and that the particular requirements implemented through software can be consistently fulfilled.

Supplier: This term is used generically and can mean the manufacturer, a vendor, a service agent, or a consultant, depending on the circumstances.

⟨1059⟩ EXCIPIENT PERFORMANCE

INTRODUCTION

Excipients are used in virtually all drug products and are essential for product manufacturing and performance. Thus, the successful manufacture of a robust product requires the use of well-defined excipients and manufacturing processes that consistently yield a quality product. Excipients used in drug products typically are manufactured and supplied in compliance with compendial standards. However, the effects of excipient properties on the critical quality attributes (CQAs) of a drug product are unique for each formulation and process and may depend on properties of excipients that are not evaluated in *USP* or *NF* monographs. The effects of variations in excipient material attributes depend on the role of an excipient in a formulation and the CQAs of the drug product. This general chapter provides a framework for applying Quality by Design (QBD) principles to excipient quality and performance.

An excipient may be used in different ways or for different purposes in a formulation and may therefore require different material attributes to achieve the desired performance. Excipient functional categories are broad, qualitative, and descriptive terms for the purpose an excipient serves in a formulation. A list of excipients grouped by functional category is included in *NF* under *Front Matter, Excipients,* which summarizes some of the more common purposes that excipients serve in drug products. Also important are the material attributes of the ingredients that must be identified and controlled to ensure the excipient performs its intended function in a drug product. A critical material attribute (CMA) is a physical, chemical, biological, or microbiological property of a material that must be within an appropriate limit, range, or distribution to ensure that drug product CQAs are maintained throughout the product life cycle. Most, but not all, CMAs become tests in a compendial monograph. In some applications, excipient suppliers and users will need to identify and control material attributes in addition to monograph specifications. Identification of CMAs requires a thorough understanding of drug product CQAs; the manufacturing process(es); and the physical, chemical, biological, or microbiological properties of each ingredient. Manufacturers should anticipate lot-to-lot and supplier-to-supplier variability in excipient properties and should have in place appropriate control measures to ensure that CMAs are maintained within the required limits. Prior knowledge, experimental designs, and risk-assessment tools can be used to prioritize and identify CMAs of excipients as well as critical process parameters. A CMA of an excipient may not be related to the major component of the excipient because, for example, the presence of minor components (e.g., peroxides, elemental impurities, or microbiological content) may affect product stability or quality. Good product development practices, which at times are termed QBD principles, require understanding excipient CMAs that contribute to consistent performance and are the foundation of a control strategy that accommodates excipient variability, consistently achieving final product CQAs.

This informational general chapter provides an overview of the key functional categories of excipients and tests or procedures that can be used to monitor and control CMAs.[1]

In this chapter, the functional categories have been organized by their most typical use in common pharmaceutical dosage forms. However, functional categories can apply to multiple dosage forms. The association of a functional category with a particular dosage form does not limit the use of an excipient to a single type of dosage form or delivery system. Each functional category includes a general description; the mechanisms by which excipients achieve their function; physical properties common to these excipients; chemical properties; and a list of *USP* general chapters that can be useful in the development of specific tests, procedures, and acceptance criteria to ensure that CMAs are adequately monitored and controlled. Because of the complex nature and interplay of formulation ingredients, processing, and dosage form performance requirements, the information provided in this chapter should not be viewed as either restrictive or completely comprehensive.

Change to read:

TABLETS AND CAPSULES

Functional Category: Diluent

DESCRIPTION

Diluents are components that are incorporated into tablet or capsule dosage forms to increase dosage form volume or weight. Sometimes referred to as fillers, diluents often compose a large portion of the dosage form, and the quantity and type of diluent

[1] This general information chapter provides nonmandatory information that does not create compendial requirements. For additional information about nonmandatory general chapters and alternative methods and procedures, see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures.*

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selected often depend on its physical and chemical properties. Thus, successful and robust manufacturing and dosage form performance depend on the measurement and control of the CMAs.

FUNCTIONAL MECHANISM

Among the most important functional roles diluents play is their ability to impart desirable manufacturing properties (e.g., powder flow, tablet compaction strength, wet or dry granule formation, or homogeneity) and performance (e.g., content uniformity, disintegration, dissolution, tablet integrity, friability, or physical and chemical stability). Some diluents (e.g., microcrystalline cellulose) occasionally are referred to as "dry binders" because of the high degree of tablet strength they impart to the final compressed tablet.

PHYSICAL PROPERTIES

The primary physical properties relevant to tablet/capsule diluents are those that can have a direct effect on diluent and formulation performance. These include: 1) particle size and size distribution, 2) particle shape, 3) bulk/tapped/true density, 4) specific surface area, 5) crystallinity, 6) moisture content, 7) powder flow, 8) solubility, 9) crystal form, and 10) compaction properties for tablet dosage forms.

CHEMICAL PROPERTIES

Tablet diluents comprise a large and diverse group of materials that include inorganics (e.g., dibasic calcium phosphate or calcium carbonate), single-component organic materials (e.g., lactose monohydrate or mannitol), and multicomponent (e.g., silicified microcrystalline cellulose or sugar spheres), or complex organics (e.g., microcrystalline cellulose or starch). They may be soluble or insoluble in water, and they may be neutral, acidic, or alkaline in nature. These chemical properties can have a positive or negative effect on the drug substance physical or chemical stability and on performance. Appropriate selection of excipients with desirable physical and chemical properties can enhance the physical and chemical stability as well as the performance of the drug substance and dosage form. The detailed composition of an excipient may be important because excipient function could be influenced by the presence of minor concomitant components that are essential for proper performance. Pharmaceutical scientists may find it necessary to control the presence of undesirable components (e.g., elemental impurities or peroxides) to ensure adequate dosage form stability and performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in diluent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Powder Fineness* ⟨811⟩, *Specific Surface Area* ⟨846⟩, *Water Determination* ⟨921⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: Wet Binder

DESCRIPTION

Tablet and capsule binders are incorporated into formulations to facilitate the agglomeration of powder into granules during mixing with a granulating fluid such as water, hydroalcoholic mixtures, or other solvents. The binder may be either dissolved or dispersed in the granulation liquid or blended in a dry state, and other components and the granulation liquid may be added separately during agitation. Following evaporation of the granulation liquid, binders typically produce dry granules that achieve the desired properties such as granule size, size distribution, shape, content, mass, and active content. Wet granulation facilitates the further processing of the granules by improving one or more of the granule properties such as flow, handling, strength, resistance to segregation, dustiness, appearance, solubility, compaction, or drug release.

FUNCTIONAL MECHANISM

Binders are soluble or partially soluble in the granulating solvent or, as in the case of native starches, can be made soluble. Concentrated binder solutions also have adhesive properties. Upon addition of liquid, binders typically facilitate the production of moist granules (agglomerates) by altering interparticle adhesion. They also may modify interfacial properties, viscosity, or other properties. During drying they may produce solid bridges that yield improved residual dry granule strength.

PHYSICAL PROPERTIES

Dispersion or dissolution of a binder in the granulation liquid depends on its physical properties: depending on the application, then surface tension, particle size, size distribution, solubility, and viscosity are among the important properties. Homogeneous incorporation of a binder into a dry blend also depends on its physical properties such as particle size, shape, and size distribution. Viscosity often is an important property to consider for binders: for polymers, viscosity is influenced by the nature of the polymer structure, molecular weight, and molecular weight distribution. Polymeric binders may form gels.

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CHEMICAL PROPERTIES

Tablet and capsule binders can be categorized as: 1) natural polymers, 2) synthetic polymers, or 3) sugars. The chemical nature of polymers—including polymeric structure, monomer properties and sequence, functional groups, degree of substitution, and cross-linking—influences the complex interactions that can occur during granulation. Natural polymers in particular may exhibit greater variation in their properties because of variations in their sources and therefore their composition.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in binder functions: *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Crystallinity* ⟨695⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Viscosity—Capillary Methods* ⟨911⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: Disintegrant

DESCRIPTION

Disintegrants are functional components that are added to formulations to promote rapid disintegration into smaller units and to allow a drug substance to dissolve more rapidly. Disintegrants are natural, synthetic, or chemically modified natural polymeric substances. When disintegrants come in contact with water or stomach or intestinal fluid, they function by absorbing liquid and start to swell, dissolve, or form gels. This causes the tablet structure to rupture and disintegrate, producing increased surfaces for enhanced dissolution of the drug substance.

FUNCTIONAL MECHANISM(S)

The ability to interact strongly with water is essential to the disintegrant function. Three major mechanisms describe the function of the various disintegrants: volume increase by swelling, deformation, and capillary action (wicking). In tablet formulations, the function of disintegrants is best described as a combination of two or more of these effects. The onset and degree of the locally achieved actions depend on various parameters of a disintegrant, such as its chemical nature and its particle size distribution and particle shape, as well as some important tablet parameters such as hardness and porosity.

PHYSICAL PROPERTIES

The primary physical properties relevant to a disintegrant are those that describe the product's particle structure as a dry powder or its structure when in contact with water. These properties may include: 1) particle size distribution; 2) water absorption rate; 3) swelling ratio or swelling index; and 4) the characterization of the resulting product, whether it is still a particulate or a gel is formed.

CHEMICAL PROPERTIES

Polymers used as disintegrants are either nonionic or anionic with counterions such as sodium, calcium, or potassium. Nonionic polymers are natural or physically modified polysaccharides such as starches, celluloses, pullulan, or cross-linked polyvinylpyrrolidone. The anionic polymers mainly are chemically modified starches, cellulose products, or low-cross-linked polyacrylates. These chemical properties should be considered in the case of ionic polymers. Disintegration performance is affected by pH changes in the gastrointestinal tract or by complex formation with ionic drug substances.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in disintegrant functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: Lubricant

DESCRIPTION

Lubricants typically are used to reduce the frictional forces between particles and between particles and metal-contact surfaces of manufacturing equipment such as tablet punches and dies used in the manufacture of solid dosage forms. Liquid lubricants may be absorbed into the granule matrix before compaction. Liquid lubricants also can be used to reduce metal–metal friction on manufacturing equipment.

FUNCTIONAL MECHANISM

Boundary lubricants function by adhering to solid surfaces (granules and machine parts) and by reducing the particle–particle friction or the particle–metal friction. The orientation of the adherent lubricant particles is influenced by the properties of the substrate surface. For optimal performance, the boundary lubricant particles should be composed of small, plate-like crystals or stacks of plate-like crystals. Fluid film lubricants melt under pressure and thereby create a thin fluid film around particles and on the surface of punches and dies in tablet presses, which helps to reduce friction. Fluid film lubricants resolidify after the

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pressure is removed. Liquid lubricants are released from the granules under pressure and create a fluid film. They do not resolidify when the pressure is removed but are reabsorbed or redistributed through the tablet matrix over the course of time.

PHYSICAL PROPERTIES

The physical properties that are important for the function of boundary lubricants include particle size, surface area, hydration state, and polymorphic form. Purity (e.g., stearate:palmitate ratio) and moisture content also may be important. The physical properties of possible importance for fluid film lubricants are particle size and solid state/thermal behavior. Purity also may be important.

CHEMICAL PROPERTIES

Lubricants can be classified as boundary lubricants, fluid film lubricants, or liquid lubricants. Boundary lubricants are salts of long-chain fatty acids (e.g., magnesium stearate) or fatty acid esters (e.g., sodium stearyl fumarate) with a polar head and fatty acid tail. Fluid film lubricants are solid fats (e.g., hydrogenated vegetable oil, type 1), glycerides (glyceryl behenate and distearate), or fatty acids (e.g., stearic acid) that melt when subjected to pressure. Liquid lubricants are liquid materials that are released from granules under pressure.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in lubricant functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Thermal Analysis* ⟨891⟩, *Water Determination* ⟨921⟩, and *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩.

ADDITIONAL INFORMATION

Certain lubricants, particularly those used in effervescent dosage forms, do not fall into the chemical categories defined above. These materials are used in special situations, and they are not suitable for universal application. Talc is an inorganic material that may have some lubricant properties. It is generally used in combination with fluid film lubricants to reduce sticking to punches and dies.

Functional Category: Glidant and/or Anticaking Agent

DESCRIPTION

Glidants and anticaking agents are used to promote powder flow and to reduce the caking or clumping that can occur when powders are stored in bulk. In addition, glidants and anticaking agents reduce the incidence of bridging during the emptying of powder hoppers and during powder processing.

FUNCTIONAL MECHANISM

Glidants are thought to work by a combination of adsorption onto the surface of larger particles and reduction of particle–particle adhesive and cohesive forces, thus allowing particles to move more easily relative to one another. In addition, glidants may be dispersed among larger particles and thus may reduce friction between these particles. Anticaking agents may absorb free moisture that otherwise would allow the development of particle–particle bridges that are implicated in caking phenomena.

PHYSICAL PROPERTIES

Primary physical properties of potential importance for glidants and anticaking agents are particle size, particle size distribution, and surface area. They may be slightly hygroscopic.

CHEMICAL PROPERTIES

Glidants and anticaking agents typically are finely divided inorganic materials. Typically they are insoluble in water. Some of these materials are complex.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in glidant or anticaking agent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, and *Water Determination* ⟨921⟩.

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Functional Category: Coloring Agent

DESCRIPTION

Coloring agents are incorporated into dosage forms to produce a distinctive appearance that may serve to differentiate a product from others that have a similar physical appearance or, in some instances, to protect photolabile components of the dosage form. These substances are subdivided into dyes (water-soluble substances), lakes (insoluble forms of a dye that result from its irreversible adsorption onto a hydrous metal oxide), inorganic pigments (substances such as titanium dioxide or iron oxides), and natural colorants (colored compounds not considered dyes per se, such as riboflavin). Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before its use.

The Federal Food, Drug, and Cosmetic Act defines three categories of coloring agents:

- FD&C colors: those certifiable for use in coloring foods, drugs, and cosmetics
- D&C colors: dyes and pigments considered safe in drugs and cosmetics when in contact with mucous membranes or when ingested
- Ext. D&C colors: colorants that, because of their oral toxicity, are not certifiable for use in ingestible products but are considered safe for use in externally applied products.

FUNCTIONAL MECHANISM

Water-soluble dyes usually are dissolved in a granulating fluid for use, although they also may be adsorbed onto carriers such as starch, lactose, or sugar from aqueous or alcoholic solutions. These latter products often are dried and used as formulation ingredients. Because of their insoluble character, lakes almost always are blended with other dry excipients during formulation. For this reason, direct-compression tablets often are colored with lakes.

PHYSICAL PROPERTIES

Particle size and size distribution of dyes and lakes can influence product processing times (blending and dissolution), color intensity, and uniformity of appearance. A coloring agent should be physically nonreactive with other excipients and the drug substances.

CHEMICAL PROPERTIES

The most important properties of a coloring agent are its depth of color and resistance to fading over time. Substances can be graded on their efficiency in reflecting desired colors of visible light, as well as on their molar absorptivities at characteristic wavelengths. A coloring agent should be chemically nonreactive with other excipients and the drug substances. The quality of a coloring agent ordinarily is measured by a determination of its strength, performance, or assay. The impurity profile is established by measurements of insoluble matter, inorganic salt content, metal content, and organic impurities.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected coloring agent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩ and *Color—Instrumental Measurement* ⟨1061⟩. Instrumental methods should be used to determine the absolute color of a coloring agent.

ADDITIONAL INFORMATION

Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before it is used.

Functional Category: Capsule Shell

DESCRIPTION

The word "capsule" is derived from the Latin *capsula,* which means a small container. Among other benefits, capsules enable pharmaceutical powders and liquids to be formulated for dosing accuracy, as well as ease of transportation. The capsule material should be compatible with all other ingredients in the drug product. Hard capsules typically consist of two parts: both are cylindrical, and one part is slightly longer than the other and is called the body. The cap fits closely on the body to enclose the capsule. In contrast, the soft capsule is a one-piece unit that may be seamed along an axis or may be seamless. The capsule material may be derived from hydrolysis of collagen that originates from porcine, bovine, or fish sources, or it can be of nonanimal origin, e.g., cellulosic or polysaccharide chemical entities. The capsule shell also contains other additives such as plasticizers, colorants, and preservatives. In some cases, capsule shells are sterilized to prevent microbial growth. The capsule shell is an integral part of the formulation, and therefore robust manufacturing and formulation performance depends on the measurement and control of CMAs. Capsules can be used to administer drugs by oral, rectal, vaginal, or inhalation routes.

FUNCTIONAL MECHANISM

Capsules can enclose solid, semisolid, or liquid formulations. Capsules have a variety of benefits: masking unpleasant taste, facilitating blinding in clinical studies, promoting ease of swallowing, and presenting a unique appearance. Conventional

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capsule shells should dissolve rapidly at 37° in biological fluids such as gastric and intestinal media. However, the solubility properties of the shell can be modified (e.g., with enteric and controlled-release polymers) to control the release of the capsule contents.

PHYSICAL PROPERTIES

The primary physical properties relevant to the capsule shell are those that can have a direct effect on product performance: 1) moisture content, 2) gas permeability, 3) stability on storage, 4) disintegration, 5) compactness, and 6) brittleness. The moisture content varies with the type of capsule. Hard gelatin capsules typically contain 13%–16% water compared to hypromellose (hydroxypropyl methylcellulose or HPMC) capsules that typically contain 4%–7% water content. Moisture content has an important effect on capsule brittleness. Soft gelatin capsules contain 5%–15% water. Equilibrium water content also may be crucial to dosage form stability because water migration can take place between the shell and capsule contents. Gas permeability may be important and generally is greater for HPMC capsules than for gelatin capsules because of the presence of open structures in the former. Unlike HPMC capsules, which do not cross-link, gelatin capsules have the potential to cross-link due to environmental and chemical exposure. Gelatin capsules may undergo cross-linking upon storage at elevated temperature and humidity [e.g., 40°(75% RH)]. Gelatin shell material is also well known to cross-link due to exposure to aldehydes, ketones, and certain dyes in shell formulations. Thus, presence of these materials in excipients should be considered for gelatin encapsulated products. Cross-linking slows in vitro dissolution and often necessitates introduction of enzymes in the test medium. Gelatin capsules should disintegrate within 15 min when exposed to 0.5% hydrochloric acid at 36°–38° but not below 30°. HPMC capsules can disintegrate below 30°.

CHEMICAL PROPERTIES

Gelatin is a commercial protein derived from the native protein, collagen. The product is obtained by partial hydrolysis of collagen derived from skin, white connective tissue, and bones of animals. Type A gelatin is derived by acid treatment, and Type B gelatin is derived from base treatment. The common sources of commercial gelatin are pigskin, cattle hide, cattle bone, cod skin, and tilapia skin. The gelatin capsule shell also typically contains coloring agents, plasticizers such as polyhydric alcohols, natural gums and sugars, and preservatives such as sodium metabisulfite and esters of *p*-hydroxybenzoic acid. The more commonly used nongelatin capsules today are made from HPMC. Different capsule types contain different moisture levels and may thus influence drug product stability. The detailed composition of an excipient may be important because the shell function can be influenced by small amounts of impurities in the excipients (e.g., peroxides in oils or aldehydes in lactose and starches) that can cause capsule cross-linking. The presence in capsule shells of undesirable materials, such as metals, odorants, water-insoluble substances, and sulfur dioxide, should be evaluated to ensure stability and performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Arsenic* ⟨211⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Residue on Ignition* ⟨281⟩, *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Water Determination* ⟨921⟩, and *Color—Instrumental Measurement* ⟨1061⟩.

ADDITIONAL INFORMATION

In addition to the general chapters listed above, useful information for ensuring consistency in selected capsule shell functions may be found in *Gelatin, Gel Strength (Bloom Value)*.

Functional Category: Coating Agent

DESCRIPTION

Oral tablets may be coated using compression coating, sugar coating, or film coating. Compression coating (effectively making a tablet within a tablet) typically uses the same ingredients as a conventional tablet and thus is outside the scope of this section. The term "sugar coating" refers to a process and does not require that sucrose be part of the formulation. Oral capsules can be coated using film-coating procedures. Reasons for coating pharmaceutical dosage forms include masking unpleasant tastes or odors, improving ingestion and appearance, protecting active ingredients from the environment, and modifying the release of the active ingredient (e.g., controlled-release or gastrointestinal targeting). Materials used as coating agents differ depending on the coating process used. Sugar coating was the original coating process. However, today for technical and economic reasons, sugar coating largely has been replaced by film coating. Sugar coating is a complex process that typically involves the application of several different coats including a seal coat, key coat, subcoat, smoothing coat, color coat, and polishing coat. The coating solutions or suspensions are slowly poured or otherwise applied in aliquots onto a bed of tablets in a slowly rotating pan. The coating process typically takes an extended period (potentially several days) and results in a substantial increase in tablet weight. In contrast, film coating generally is a simpler process in which coating solution or suspension is sprayed onto tablets either in a rotating pan or in a fluid-bed apparatus and results in only a modest increase in capsule or tablet weight. The materials used in both sugar coating and film coating include natural, semisynthetic, and synthetic materials. These may be solutions, suspensions, or colloidal dispersions (latexes or pseudolatexes) that can be applied as either aqueous or nonaqueous systems. In addition, waxes and lipids can be applied as coatings in the molten state without the use of solvents. They also can be applied in the solid state as a polishing coat on top of sugar coating or film coating.

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FUNCTIONAL MECHANISM—SUGAR COATING

The seal coat is used to seal the surface of the tablet cores to prevent water in the coating solutions or suspensions from causing the tablet cores to disintegrate during coating. The seal coat typically is a polymer (e.g., shellac) that is insoluble in water and is applied as a thin coat from a solution in a nonaqueous solvent. The key component of the majority of sugar-coating solutions or suspensions is a solute, typically sucrose, that is highly soluble in water and forms a sticky, viscous solution (a syrup) at very high concentration. Other materials can be dissolved or suspended in the solution, depending on the stage during the coating process. As the coating dries, the dissolved coating material adheres to the surface of the tablets. The coating solution or suspension typically is applied in incremental steps, followed by drying, until the requisite coating has been achieved. The key coat is composed of another thin coat that is designed to adhere to the seal-coated cores to provide a base for the subcoat so the latter can adhere to the tablet surface. The subcoat typically contains a high concentration of suspended solids and is designed to increase the weight of the tablets comparatively quickly. The smoothing coat is designed to provide a smooth surface, and the color coat provides the final color if required. Finally, the tablets may be transferred to a polishing pan and polished using a mixture of waxes to provide a high-gloss finish.

FUNCTIONAL MECHANISM—FILM COATING

Film-coating agents are composed of film-forming materials (see *Functional Category: Film-Forming Agent*) that impart desirable pharmaceutical properties such as appearance and patient acceptance (e.g., taste masking and ease of swallowing). Film-coating agents also can serve other functional purposes such as providing a barrier against undesirable chemical reactions or untimely release of a drug from its components. After ingestion, the film coating may dissolve by processes such as hydration, solubilization, or disintegration, depending on the nature of the material used. Enteric coatings are insoluble in acidic (low pH) media but dissolve readily in neutral pH conditions. However, most common film-coating polymers do not have pH-specific solubility. The thickness of the film may vary by application and the nature of the coating agents. In the coating process, the polymer chains spread out on the core surface and coalesce into a continuous film as the solvent evaporates. Polymer solutions or dispersions with a low viscosity and high pigment-binding capacity reduce the coating time and facilitate relatively simple and cost-effective manufacturing. Plastic polymers, waxes, and lipid-based coatings can be applied without solvents by melting and atomization. When molten fluid droplets strike the surface of the fluidized drug particles, they spread and resolidify to form film layers. Therefore, film coating materials generally have the ability to form a complete and stable film around the substrate. The film coating typically is applied uniformly and is carefully dried to ensure that a consistent product is produced. Suitable plasticizers may be required to lower the minimum film-forming temperature of the polymer, and formulators should consider their potential effect on drug release.

PHYSICAL PROPERTIES

Sugar coating is a lengthy, complex process. The physical properties of the seal-coating polymer and solution are important. The physical properties of the syrup component in the subsequent layers and any dissolved or suspended solids also are important, and coating agents must be sufficiently stable during use.

Film coating is a complex process, and the characteristics of the film-forming polymer are important. The particle size of colloidal dispersions varies with their composition and manufacture (latex, pseudolatex, or redispersed powder) and can have an effect on film formation. The surface tension of coating preparations can influence the spray pattern in the manufacturing process. The film should possess sufficient elasticity and mechanical strength to withstand the stresses during coating and packaging operations. For coatings that are applied in a molten state without solvents (plastic polymers, waxes, and lipid-based coatings), melting range and melt viscosity are the primary properties that formulators must consider.

CHEMICAL PROPERTIES

Coating components can be of natural, semisynthetic, or synthetic origin and also can be available in different chemical grades. They comprise a diverse variety of different chemical materials. Formulators must consider the nature of the material and its intended use when they identify and quantitate chemical CMAs to ensure consistent performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected coating agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Dissolution* ⟨711⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩. In addition, the general chapters listed under *Functional Category: Film-Forming Agent* (below) also may be appropriate for the evaluation of film-coating polymers.

ADDITIONAL INFORMATION

Additives often are included in a coating formulation. Fillers (e.g., sugar alcohols, microcrystalline cellulose, calcium carbonate, and kaolin) may be added to increase the solids content of the coating agent without increasing viscosity. Stearic acid can be used to improve the protective function/moisture barrier of a coating. Coloring agents (e.g., titanium dioxide and iron oxides) may be added to modify appearance.

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Functional Category: Plasticizer

DESCRIPTION

A plasticizer is a low molecular weight substance that, when added to another material—usually a polymer—makes the latter flexible, resilient, and easier to handle. Plasticizers are key components that determine the physical properties of polymeric pharmaceutical systems such as tablet film coatings and capsule shells.

FUNCTIONAL MECHANISM

Plasticizers function by increasing the intermolecular and intramolecular mobility of the macromolecules that comprise polymeric materials. They achieve this by interfering with the normal intermolecular and intramolecular bonding mechanisms in such systems. The most effective plasticizers exert their effect at low concentrations, typically less than 5% w/w. Plasticizers commonly are added to film coatings (aqueous and nonaqueous systems) and capsule shells (hard and soft varieties) to improve their workability and mechanical ruggedness. Without the addition of plasticizers, such materials can split or fracture prematurely. Plasticizers also are added to semisolid pharmaceutical preparations, such as creams and ointments, to enhance their rheological properties.

PHYSICAL PROPERTIES

The most common plasticizers are low molecular weight (<500 Da) solids or liquids. They typically have low melting points (<100°) and can be volatile (i.e., exert an appreciable vapor pressure) at ambient temperature. Plasticizers can reduce the glass transition temperature (T_g) of the system to which they are added.

CHEMICAL PROPERTIES

Many modern plasticizers are synthetic esters such as citrates and phthalates. Traditional pharmaceutical plasticizers include oils, sugars, and their derivatives.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected excipient functions: *Residual Solvents* ⟨467⟩, *Melting Range or Temperature* ⟨741⟩, *Refractive Index* ⟨831⟩, *Specific Gravity* ⟨841⟩, *Thermal Analysis* ⟨891⟩, and *Water Determination* ⟨921⟩.

ADDITIONAL INFORMATION

The choice of an appropriate plasticizer often is guided by reference to its solubility parameter, which is related to its cohesive energy density. Solubility parameter values for many common materials are tabulated in standard reference texts. To ensure maximum effectiveness, the solubility parameter of the plasticizer and the polymeric system being plasticized should be matched as closely as possible.

Functional Category: Film-Forming Agent

DESCRIPTION

Film-forming agents typically are polymers that can be used to prepare polymer films to coat tablets or capsules for oral administration, to modify appearance, to modify drug release, or to serve other purposes such as melt-in-the-mouth formulations. Polymeric materials used as film-forming agents can be derived from natural, semisynthetic, or synthetic sources, and they can be supplied as powders, granules, pre-prepared solutions, or colloidal dispersions. Colloidal dispersions may contain other components such as plasticizers, surface-active agents, preservatives, or stabilizers. Film-forming agents can be applied as colloidal dispersions (latexes or pseudolatexes) or as aqueous, hydroalcoholic, or nonaqueous polymeric solutions.

FUNCTIONAL MECHANISM

Film-forming agents typically are composed of polymeric materials that possess the ability to form films after solvent evaporation from a solution of the polymer or from the continuous phase of a colloidal dispersion. Thus, the polymer alone must be a solid at ambient temperature and humidity. Some polymers can form films without the inclusion of added components, but other polymers may require the use of additional components such as plasticizers.

PHYSICAL PROPERTIES

Many polymeric film-forming agents are available in a variety of physical grades that typically are based on the nominal viscosity of the particular grade. The physical properties of the polymer usually are those of a solid, and many polymers are available as powders and granules. In addition to the normal properties of bulk powders and granules, other important physical properties of a polymeric film-forming agent are the molecular weight distribution, which is linked to the nominal viscosity of the grade, and the glass transition temperature (T_g). If the film-forming agent is provided as a pre-prepared solution or dispersion, the viscosity of the solution or dispersion can affect performance and should be monitored.

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CHEMICAL PROPERTIES

Film-forming agents comprise a diverse group of materials, including natural, semisynthetic, and synthetic materials as discussed above. They may have ionizable functional groups that impart pH-dependent properties and also can be available in different chemical grades (e.g., with different degrees of chemical substitution). Pharmacopeial monographs often describe classes of polymeric materials that allow a considerable range of composition. Formulators should consider these factors when they identify critical material attributes and establish specifications to ensure consistent performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected film-forming agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Dissolution* ⟨711⟩, *Optical Microscopy* ⟨776⟩, *pH* ⟨791⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Bulk Powder Sampling Procedures* ⟨1097⟩, ▲*Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩▲ (CN 1-May-2020), *Raman Spectroscopy* ⟨1120⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩*Powder Flow* ⟨1174⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

Functional Category: Flavor and Fragrance

DESCRIPTION

A flavor is a single chemical entity or a blend of chemicals of natural or synthetic origin that has the ability to elicit a taste or aroma (i.e., fragrance) response when orally consumed or smelled. The primary purpose of flavor that is added to a pharmaceutical preparation is to provide all or part of the taste and aroma of the product taken into the mouth. Flavors commonly are used in pharmaceutical oral disintegrating tablets, oral solutions, and oral suspensions to mask objectionable drug taste and to make the formulation more palatable, thus promoting patient compliance.

FUNCTIONAL MECHANISM

Chemicals dissolved in saliva excite chemoreceptors on taste buds that reside primarily on the tongue and thus arouse taste perception. Dissolution also releases volatile chemicals that reach the olfactory receptors, triggering aroma perception. The total of taste and odor responses constitutes flavor. Humans can distinguish among five components of taste: sourness, saltiness, sweetness, bitterness, umami (savory), and a wide range of specific odors. Flavor enhancers and taste modifiers can be used to modify the sweetness profile of a sweetening agent or to mask off-flavors. For example, organic acids, such as aspartic and glutamic acids, are known to reduce bitterness.

PHYSICAL PROPERTIES

Taste perception depends on physicochemical, physiological, and psychological factors. Physical properties such as particle size, solubility, humectancy, texture, and color all influence the senses. In addition to flavor, the sensory attributes of sight (e.g., appealing color), sound (e.g., crunch of a chewable tablet), and mouth feel (e.g., viscous, slimy, chalky, cloying, or watery) also contribute to and influence the overall sensory experience.

CHEMICAL PROPERTIES

Chemicals that provide one of the five basic tastes possess a wide variety of structures, functional groups, and molecular weights. Chemicals used to flavor pharmaceuticals by providing both odor and taste tend to have low molecular weights (<250 Da) and polar functional groups such as esters, ketones, aldehydes, amines, or alcohols. To increase the stability of the flavor(s) in a solid dosage form and to minimize flavor–drug interactions, formulators can add flavors in an encapsulated or spray-dried form.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in flavor functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Chromatography* ⟨621⟩, *Congealing Temperature* ⟨651⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Optical Rotation* ⟨781⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Refractive Index* ⟨831⟩, and *Specific Gravity* ⟨841⟩.

Functional Category: Release-Modifying Agents

Release-modifying agents are used to control drug release in extended-release formulations (also referred to as prolonged-release or controlled-release formulations). Sustained-release and enteric coating agents are included under *Functional Category: Coating Agent.*

DESCRIPTION

Release-modifying agents change a medicinal product's drug-release pattern to achieve the desired drug plasma profile for a given time. The majority of release-modifying agents are polymers that differ in solubility, ease of erosion, rate of swelling, or

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sensitivity to the biological environment in which they are placed. These polymers have been used to fabricate matrix- or membrane-based drug delivery systems for oral, parenteral, transdermal, and other routes of administration. Matrix controlled-release drug delivery systems can be classified as hydrophilic eroding matrices, hydrophilic noneroding matrices, or hydrophobic matrices. In membrane controlled-release drug delivery systems, the drug reservoir is coated by a rate-controlling polymeric membrane that may consist of a blend of polymers to control release. Such devices may take the form of tablets, capsules, microspheres, vesicles, fibers, patches, and others. In addition to polymers, certain lipid-based excipients also can be used as release-modifying agents in hydrophobic matrix devices and other types of modified-release systems. Typically, these lipid-based materials are fats and waxes or related materials with melting ranges above 45°.

FUNCTIONAL MECHANISM

Upon contact with a biological fluid, release-controlling polymers may undergo a variety of physical changes such as swelling, gelling, dissolution, or erosion, each of which can be triggered by simple aqueous exposure or can be modulated by pH, osmotic stress, or interactions with bile or other intestinal contents. In addition to physical changes, polymers may undergo chemical degradation by acids, bases, enzymes, water, heat, and others. Any or all of these mechanisms may act in concert to control the rate at which the drug is released from the delivery system.

For hydrophilic matrices in which drug diffusion dominates release rate, the rate of drug release depends on the properties of the polymer gel and the nature of the continuous phase in the interstices of the gel influences the dissolution and diffusion rates of the drug. In the case of eroding matrices, the gel erodes because of the mechanical action of the gastrointestinal tract as the water uptake increases, and the gel becomes more dilute, thus reducing the diffusion distance or releasing drug particles that subsequently dissolve. Hydrophobic matrix-forming materials are not soluble. Drug release from such systems is governed by drug diffusion through the tortuous pores that remain as soluble components dissolve.

Membrane-based drug delivery systems include polymer-coated tablets, capsules, and microspheres. Drug-release mechanisms from such systems are complex and depend on physicochemical characteristics of the drug and polymers or lipids used as well as biological factors in the case of biocompatible and biodegradable systems. Most commonly, drug release from such systems is governed by drug diffusion through the hydrated rate-controlling membrane.

Other modified-release systems for parenteral use include solid lipid nanoparticles and liposomes. The release mechanisms for these systems often involve a complicated interplay with biological processes such as potential clearance through the reticulo-endothelial system, targeted delivery, and cellular uptake.

Osmotic pump devices are a special case of membrane delivery systems. The rate-controlling polymer is insoluble and semipermeable—i.e., it will allow water but not drug molecules—to diffuse through the membrane. Release is controlled by the osmotic pressure of the core components and the viscosity of the resulting solution or suspension. The drug, either in solution or as a suspension, is forced out of a hole in the membrane, which is typically drilled by a laser during product manufacture.

PHYSICAL PROPERTIES

The physical properties of the release-controlling excipient depend on the chemical type: hydrophilic polymer, hydrophobic polymer, semipermeable polymer blends, or lipid, wax, or biodegradable polymer (which can be hydrophilic or hydrophobic).

Hydrophilic polymers gel in contact with water or aqueous media. Because they should provide resistance to the mechanical action of the gastrointestinal tract during passage, they typically are higher molecular weight grades of the polymers. At the concentrations typically used during in vivo release, these high molecular weight polymers often do not exhibit Newtonian properties except in very dilute solution (if they are soluble). Formulators should monitor the kinetic and viscoelastic properties of the gels formed in the release medium.

Hydrophobic polymers are insoluble in water, and their solution properties are determined in nonaqueous solutions. The polymers used in the preparation of semipermeable membranes in osmotic pump devices also are insoluble in water, and similarly their solution properties are determined in nonaqueous solutions. Similarly, hydrophobic lipid-based materials are insoluble in water.

CHEMICAL PROPERTIES

Release-controlling agents have many different types and origins and are available in a range of grades that reflect the considerable variation in their chemical structures and properties. Formulators must consider these variables during any chemical investigation and when they consider the effects of chemical structure on excipient performance. Properties of interest may include chemical composition for copolymers and cellulosic derivatives, degree of ionization, molecular weight, degree of cross-linking, or, for lipids, fatty acid composition. Residual impurities from the manufacturing process, e.g., monomers, initiators, quenching agents, peroxides, and aldehydes, may affect drug substance stability and should be monitored.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected functions of release-modifying agents: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Dissolution* ⟨711⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Water Determination* ⟨921⟩, *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩, *Powder Flow* ⟨1174⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

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ADDITIONAL INFORMATION

Some release-modifying agents may include additives such as an antioxidant or an anticaking agent.

Change to read:

ORAL LIQUIDS

Functional Category: pH Modifier (Acidifying/Alkalizing/Buffering Agent)

DESCRIPTION

The hydrogen ion concentration, $[H^+]$, in an aqueous solution is expressed as $pH = -\log(H^+)$. The pH of pure water is 7 at 25°. An aqueous solution is acidic at pH <7 and alkaline at pH >7. An acid can be added to acidify a solution. Similarly, a base can be used to alkalize a solution. A buffer is a weak acid (or base) and its salt. When a buffer is present in a solution, the addition of small quantities of strong acid or base leads to only a small change in solution pH. Buffer capacity is influenced by salt/acid (or base/salt) ratio and total concentration of acid (or base) and salt. The pH of pharmaceutical solutions typically is controlled using acidifying/alkalizing and buffering agents to: 1) maintain a pH close to that of relevant body fluid to avoid irritation, 2) improve drug stability where it is found to be pH dependent, 3) control equilibrium solubility of weak acids or bases, and 4) maintain a consistent ionization state of molecules during chemical analysis, e.g., high-performance liquid chromatography.

FUNCTIONAL MECHANISM

The ionization equilibria of weak bases, weak acids, and water are the key to the functions of acidifying, alkalizing, and buffering agents. The autoprotolytic reaction of water can be expressed as:

$$H_2O + H_2O \leftrightarrow H_3O^+ + OH^-$$

The autoprotolysis constant (or ion product) of water is $K_w = 1 \times 10^{-14}$ at 25° and varies significantly with temperature. Because the concentrations of hydrogen and hydroxyl ions in pure water are equal, each has the value of approximately 1×10^{-7} mol/L, leading to the neutral pH of 7 at 25°. When an acid, base, or salt of a weak acid (or base) is added, the ionization equilibrium of water is shifted so that $[H^+][OH^-]$ remains constant, thus resulting in a solution pH that is different from 7.

PHYSICAL PROPERTIES

pH modifiers typically are dissolved in liquid dosage forms. Physical properties may be important and should be considered because they may influence processing requirements (e.g., particle size may influence mixing time required to dissolve a pH modifier).

CHEMICAL PROPERTIES

Buffers and pH modifiers influence solution pH, buffer capacity, osmolality, osmolarity, and water conductivity. When used in chemical analysis, buffers must be chemically compatible with the reagents and test substance. Buffers used in physiological systems should not interfere with the pharmacological activity of the medicament or the normal function of the organism.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected pH-modifier or buffering-agent functions: *Water Conductivity* ⟨645⟩, *Osmolality and Osmolarity* ⟨785⟩, and *pH* ⟨791⟩.

Functional Category: Wetting and/or Solubilizing Agent

DESCRIPTION

Solubilizers can be used to dissolve otherwise insoluble molecules. They function by facilitating spontaneous phase transfer to yield a thermodynamically stable solution. A number of solubilizers are available commercially. Acceptable solubilizers for pharmaceutical applications have been fully evaluated in animals for safety and toxicology. Wetting agents increase the spreading and penetrating properties of a liquid by lowering its surface tension.

FUNCTIONAL MECHANISM

Wetting and solubilizing agents comprise a variety of different chemical structures or classes. Some solubilizers have unique chemical structures. For example, a hydrophilic moiety may be tethered with a hydrophobic moiety to yield distinct micelle shapes and morphologies in water, thus facilitating solubilization. The mechanism of solubilization often is associated with a favorable interaction of the insoluble agent and the interior core of the solubilizer assembly (e.g., micelles). In other cases, unique hydrophobic sites that are capable of forming inclusion complexes are present. Other types of solubilizers use a range of polymeric chains that interact with hydrophobic molecules to increase solubility by dissolving the insoluble agent into the polymeric chains.

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PHYSICAL PROPERTIES

Wetting and solubilizing agents are typically solid, liquid, or waxy materials. Their physical properties depend on their chemical structures. The physical properties and performance of the wetting agents and solubilizers, however, depend on the surface-active properties of the solubilizers and on the hydrophilic–lipophilic balance (HLB). Materials with lower HLB values behave as emulsifiers, whereas those with higher HLB values typically behave as solubilizing agents. For example, the commonly used wetting and solubilizing agent sodium lauryl sulfate (HLB 40) is hydrophilic and highly water soluble and, upon dispersion in water, spontaneously reduces surface tension and forms micelles that function to solubilize lipophilic materials.

The unique hydrophilicity and hydrophobicity properties of solubilizers can be characterized by their chemical structures, aggregate numbers, or critical micelle concentrations (CMCs). The CMC value is unique to an individual solubilizer that bears hydrophilic, lipophilic, and/or hydrophobic chains. CMC is a measure of the concentration at which the surface-active molecules aggregate. These molecular aggregates can solubilize the solute by incorporating part into the hydrophobic interior. Such interactions with the insoluble molecule further stabilize the molecules in the entire assemblies without precipitation.

CHEMICAL PROPERTIES

The chemical and surface-active properties depend on the structures of the solubilizers. Because of the complex nature of solute–solvent–solubilizer interactions, pharmaceutical scientists must carefully consider, identify, and control the CMAs of solubilizers.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected solubilizing agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *pH* ⟨791⟩, *Specific Gravity* ⟨841⟩, *Specific Surface Area* ⟨846⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

Functional Category: Antimicrobial Preservative

DESCRIPTION

Antimicrobial preservatives are used to kill or prevent growth of bacteria, yeast, and mold in the dosage form.

FUNCTIONAL MECHANISM

Preservatives work by a variety of mechanisms to control microbes. Most work at the cell membrane, causing membrane damage and cell leakage. Other modes of action include transport inhibition, protein precipitation, and proton-conducting uncoupling. Some preservatives are bactericidal (kill bacteria or yeast and mold); some are bacteriostatic (inhibit growth of microorganisms); and others are sporicidal (kill spores). Several of the preservatives can act synergistically (e.g., combinations of parabens).

PHYSICAL PROPERTIES

Antimicrobials generally are soluble in water at concentration ranges at which they are effective. The vapor pressure of these agents is important, especially if the dosage form is intended for lyophilization or spray drying. Several of these agents are flammable. Understanding an excipient's partition coefficient is important because partitioning of a preservative into an oil phase can diminish the preservative's concentration in the aqueous phase, which in turn can reduce its value as a preservative.

CHEMICAL PROPERTIES

Phenolic preservatives can undergo oxidation and color formation. Incompatibilities of preservatives (cationic and anionic mixtures, adsorption to tubes or filters, or binding to surfactants and proteins) should be taken into account during product development.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected antimicrobial functions: *Injections and Implanted Drug Products* ⟨1⟩, *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Antimicrobial Agents—Content* ⟨341⟩.

ADDITIONAL INFORMATION

Safety and labeling requirements regarding the use of antimicrobial preservatives should be considered.

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Functional Category: Chelating and/or Complexing Agents

DESCRIPTION

Chelating agents form soluble complex molecules with certain metal ions (e.g., copper, iron, manganese, lead, and calcium) and essentially remove the ions from solution to minimize or eliminate their ability to react with other elements and/or to precipitate. The agents are used in pharmaceuticals (oral, parenteral, and topical formulations), cosmetics, and foods to sequester ions from solution and to form stable complexes. Chelating agents also are referred to as chelants, chelators, or sequestering agents.

Complexing agents form soluble complex molecules (e.g., inclusion complexes) with other solutes (e.g., drug substances) and can influence physical and chemical properties such as solubility and stability.

FUNCTIONAL MECHANISM

Chelating agents are used to sequester undesirable metal ions from solution. The chemical structure of chelating agents allows them to act as a "claw" to associate with the metal atom by forming a heterocyclic ring structure. Complexing agents function similarly but mechanistically and do not (by definition) require a two-point claw structure because they can associate via one or more binding sites. All chelating agents are complexing agents, but not all complexing agents are chelating agents. Chelating agents are used as antioxidant synergists, antimicrobial synergists, and water softeners. By sequestering metal ions from solution, chelating agents reduce the propensity for oxidative reactions. Chelating agents also have the ability to enhance antimicrobial effectiveness by forming a metal-ion–deficient environment that otherwise could feed microbial growth.

Complexing agents generally form soluble complex molecules with solutes (e.g., drug molecules) that can influence physical, chemical, and drug delivery properties. Complexing agents that form inclusion complexes typically contain a hydrophobic cavity into which a drug substance can enter and an outer, more hydrophilic region.

PHYSICAL PROPERTIES

Chelating and complexing agents generally are soluble in water and typically are dissolved in liquid dosage forms. Physical properties may be important and should be considered because they may influence processing requirements (e.g., particle size may influence mixing time required to dissolve). Chelating and complexing agents exhibit different degrees of hygroscopicity. Because chelating agents are used in low levels, particle size distribution can be important to enable acceptable dosage form content uniformity.

CHEMICAL PROPERTIES

Chelating agents complex with metal ions via any combination of ionic and covalent bonds. Dilute aqueous solutions may be neutral, acidic, or alkaline. Edetic acid and its salts are incompatible with strong oxidizers, strong bases, and polyvalent metal ions (e.g., copper and nickel). Specific agents are selected for a formulation based on their solubility, affinity for the target metal ion, and stability. Edetate salts are more soluble than the free acid. Unlike other edetate salts and the free acid, edetate calcium disodium does not sequester calcium and therefore is preferred to prevent hypocalcemia. It is also preferred to chelate metals with the release of calcium ions. Alternatively, disodium edetate can be used to treat hypercalcemia. Edetic acid will decarboxylate if heated above 150°.

Complexing agents generally form molecular complexes with drug substances that are dependent on complexing agent physical and chemical properties. The ability of a solute to form an inclusion complex is dependent on complexing agent molecular weight, chemical structure, and the dimensions of the hydrophobic cavity.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected chelating and complexing functions: *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Iron* ⟨241⟩, *Lead* ⟨251⟩, *Antimicrobial Agents—Content* ⟨341⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *pH* ⟨791⟩, *Water Determination* ⟨921⟩, and ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020).

Functional Category: Antioxidant

DESCRIPTION

This category applies to antioxidants used as in vitro stabilizers of pharmaceutical preparations to mitigate oxidative processes. Antioxidants used for their biological activity in vivo may be regarded as active ingredients with therapeutic effects and are not discussed. Antioxidants delay the onset of and/or significantly reduce the rate of complex oxidative reactions that could otherwise have a detrimental effect on the drug substance. Antioxidants also can be considered for protecting nonactive components such as unsaturated oils, pegylated lipids, flavors, and essential oils. Thus, antioxidants preserve the overall integrity of the dosage form against oxidative stress. Antioxidants are most effective when incorporated in the formula to prevent or delay the onset of chain reactions and to inhibit free radicals and hydroperoxides from engaging in the cascading processes described above. Effective application of antioxidants and evaluation of their efficacy necessitate an understanding of oxidative mechanisms and the nature of the byproducts they generate. Autoxidation is initiated when oxygen reacts with a substrate to form highly reactive species known as free radicals ($RH \rightarrow R\cdot$). After "initiation" the free radicals in the presence of oxygen can

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trigger chain reactions ($R\cdot + O_2 \rightarrow ROO\cdot$ and $ROO\cdot + RH \rightarrow R\cdot + ROOH$) to form peroxy radicals, hydroperoxides, and new alkyl radicals that can initiate and then propagate their own chain reactions. The cascading reactions during the propagation phase can be accelerated by heat, light, and metal catalysts. In the presence of trace amounts of metal catalysts (Cu^{+}, Cu^{2+}, Fe^{2+}, and Fe^{3+}), hydroperoxides (ROOH) readily decompose to $RO\cdot$ and $ROO\cdot$ and subsequently can trigger reactions with the API and/or the excipients (e.g., hydrocarbons) to form hydroxyl acids, keto acids, and aldehydes that can have further undesirable effects. Note that hydroperoxides are not solely the reaction products of oxidative mechanisms within a formulation. Residual amounts of hydroperoxides also can be found in commonly used excipients like polyethylene glycols, polyvinylpyrrolidone, and polysorbates. The initiation phase generally is slow and has a limited effect on the quality of the finished product. The propagation phase, in contrast, involves rapid, irreversible degradation of chemical species.

FUNCTIONAL MECHANISM

Antioxidants can be grouped by their mode of action. Phenolic antioxidants that block free radical chain reactions also are known as true or primary antioxidants. This group consists of monohydroxy or polyhydroxy phenol compounds with ring substitutions. They have very low activation energy to donate hydrogen atom(s) in exchange for the radical electrons that are rapidly delocalized by free radicals. By accepting the radical electrons, they stabilize free radicals. The reaction yields antioxidant free radicals that also can react with lipid free radicals to form other stable compounds. Thus, they can block oxidative chain reactions both in the initiation and propagation stages. Because of their solubility behavior, phenolic antioxidants are most effective in protecting oils and oil-soluble actives against oxidative stress. Reducing agents generally are water-soluble antioxidants (e.g., L-ascorbic acid) with lower redox potential than the drug or the excipient they are protecting. They delay the onset and the rate of oxidative reactions by sacrificially reacting with oxygen and other reactive species. The oxygen-scavenging potential of the reducing agents may be sensitive to pH and also can be negatively affected in the presence of trace elements. Chelating agents bind with free metals (Cu^{+}, Cu^{2+}, Fe^{2+}, and Fe^{3+}) that may be present in trace amounts in the formulation. The newly formed complex ions are nonreactive. Chelating agents therefore remove the capacity of the metal catalysts to participate in oxidative reactions that occur during the propagation stage.

The utility of antioxidants can be maximized by synergistic use of one or two primary antioxidants along with reducing and chelating agents. The combined effect often is greater than the sum of the individual effects of each antioxidant (synergistic effect).

PHYSICAL PROPERTIES

Solubility of the antioxidant should be greatest in the formulation phase (oily, aqueous, or emulsion interface), where the drug substance is most soluble. The temperature at which the antioxidant decomposes is critical for autoclaved preparations, where loss of antioxidant activity may occur. Stability of the antioxidant also must be considered and may be a function of pH and processing conditions. Metal ions may react with propyl gallate to form colored complexes. At alkaline pH, certain proteins and sodium salts may bring about discoloration of *tert*-butylhydroquinone.

CHEMICAL PROPERTIES

Activation energy, oxidation–reduction potential, and stability at different formulation (e.g., pH) and processing (e.g., heat) conditions are important chemical properties. If the dosage form's expected shelf life depends on the antioxidant's function, the concentration must be factored in and periodically assessed to ensure that a sufficient amount of antioxidant remains throughout the product shelf life.

GENERAL CHAPTERS

The following general chapters may be useful for assessing selected excipient antioxidant functions: *Iron* ⟨241⟩, *Chromatography* ⟨621⟩, *Crystallinity* ⟨695⟩, *Melting Range or Temperature* ⟨741⟩, *Specific Surface Area* ⟨846⟩, and *Water Determination* ⟨921⟩.

Functional Category: Sweetening Agent

DESCRIPTION

Sweetening agents are used to sweeten oral dosage forms and to mask unpleasant flavors. See *Functional Category: Flavor and Fragrance* for more details.

FUNCTIONAL METABOLISM

Sweetening agents bind to receptors on the tongue that are responsible for the sensation of sweetness. The longer the sweetener molecule remains attached to the receptor, the sweeter the substance is perceived to be. The standard for sweetness is sucrose.

PHYSICAL PROPERTIES

The primary physical properties relevant to sweeteners relate to their compatibility with the other ingredients in the formulation (e.g., acidic ingredients), processing conditions (e.g., heating), particle size and distribution, moisture content, isomerism, sweetness, and taste-masking capability. These properties may be formulation dependent.

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CHEMICAL PROPERTIES

Sweeteners can be divided into three main groups: sugars (which have a ring structure), sugar alcohols (sugars that do not have a ring structure), and artificial sweeteners. All sweeteners are water soluble. The stability of many sweeteners is affected by pH and other ingredients in the formulation. Some sweeteners may catalyze the degradation of some active ingredients, especially in liquids and in cases in which the manufacturing processes involve heating.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected sweetening functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Optical Rotation* ⟨781⟩, and *Water Determination* ⟨921⟩.

ADDITIONAL INFORMATION

Products that contain aspartame must include a warning on the label stating that the product contains phenylalanine. Sugar alcohols have a glycemic index well below that of glucose. However, sorbitol is slowly metabolized to fructose and glucose, which raises blood sugar levels. Sugar alcohols in quantities generally greater than 20 g/day act as an osmotic laxative, especially when they are contained in a liquid formulation. Preservative systems should be carefully chosen to avoid incompatibility with the sweetener, and some sweeteners are incompatible with certain preservatives.

SEMISOLIDS, TOPICALS, AND SUPPOSITORIES

Functional Category: Suppository Base

DESCRIPTION

Suppository bases are used in the manufacture of suppositories (for rectal administration) and pessaries (for vaginal administration). They can be hydrophobic or hydrophilic.

FUNCTIONAL MECHANISM

Suppositories should melt at just below body temperature (37°), thereby allowing the drug to be released either by erosion and partition if the drug is dissolved in the base or by erosion and dissolution if the drug is suspended in the base. Hard fat suppository bases melt at approximately body temperature. Hydrophilic suppository bases also melt at body temperature and typically also dissolve or disperse in aqueous media. Thus, release takes place via a combination of erosion and dissolution.

PHYSICAL PROPERTIES

The important physical characteristic of suppository bases is melting range. In general, suppository bases melt between 27° and 45°. However, individual bases usually have a much narrower melting range within these temperature boundaries, typically 2°–3°. The choice of a particular melting range is dictated by the influence of the other formulation components on the melting range of the final product.

CHEMICAL PROPERTIES

Hard fat suppository bases are mixtures of semisynthetic triglyceride esters of longer-chain fatty acids. They may contain varying proportions of mono- and diglycerides and also may contain ethoxylated fatty acids. They are available in many different grades that are differentiated by melting range, hydroxyl number, acid value, iodine value, solidification range, and saponification number.

Hydrophilic suppository bases are mixtures of hydrophilic semisolid materials that in combination are solid at room temperature and yet release the drug by melting, erosion, and dissolution when administered to the patient. Hydrophilic suppository bases have much higher levels of hydroxyl groups or other hydrophilic groups than do hard fat suppository bases. Polyethylene glycols that show appropriate melting behavior are examples of hydrophilic suppository bases.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected suppository base functions: *Fats and Fixed Oils* ⟨401⟩, *Congealing Temperature* ⟨651⟩, *Melting Range or Temperature* ⟨741⟩, and *Pharmaceutical Dosage Forms* ⟨1151⟩.

ADDITIONAL INFORMATION

Some materials included in suppositories based on hard fats have much higher melting ranges. These materials typically are microcrystalline waxes that help stabilize molten suspension formulations. Suppositories also can be manufactured from glycerinated gelatin.

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Functional Category: Suspending and/or Viscosity-Increasing Agents

DESCRIPTION

Suspending and viscosity-increasing agents are used in pharmaceutical formulations to stabilize dispersal systems (e.g., suspensions or emulsions), to reduce the rate of solute or particulate transport, or to decrease the fluidity of liquid formulations.

FUNCTIONAL MECHANISMS

A number of mechanisms contribute to the dispersion stabilization or viscosity-increasing effect of these agents. The most common is the increase in viscosity—because of the entrapment of solvent by macromolecular chains or clay platelets—and the disruption of laminar flow. Other mechanisms include gel formation via a three-dimensional network of excipient molecules or particles throughout the solvent continuum and steric stabilization wherein the macromolecular or mineral component in the dispersion medium adsorbs to the surfaces of particles or droplets of the dispersed phase. The latter two mechanisms increase formulation stability by immobilizing the dispersed phase.

PHYSICAL PROPERTIES

Each of the mechanisms—increased viscosity, gel formation, or steric stabilization—is a manifestation of the rheological character of the excipient. Because of the molecular weights and sizes of these excipients, the rheological profiles of their dispersions are non-Newtonian. Dispersions of these excipients display viscoelastic properties. The molecular weight distribution and polydispersity of the macromolecular excipients in this category are important criteria for their characterization.

CHEMICAL PROPERTIES

The majority of the suspending and viscosity-increasing agents are: 1) hydrophilic carbohydrate macromolecules (acacia, agar, alginic acid, carboxymethylcellulose, carrageenans, dextrin, gellan gum, guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hypromellose, maltodextrin, methylcellulose, pectin, propylene glycol alginate, sodium alginate, starch, tragacanth, and xanthan gum); and 2) noncarbohydrate hydrophilic macromolecules, including gelatin, povidone carbomers, polyethylene oxide, and polyvinyl alcohol. Minerals (e.g., attapulgite, bentonite, magnesium aluminum silicate, and silicon dioxide) comprise the second-largest group of suspending and viscosity-increasing agents. Aluminum monostearate is the one non-macromolecular, nonmineral excipient in this functional category. It consists chiefly of variable proportions of aluminum monostearate and aluminum monopalmitate.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected viscosity-increasing functions: *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

Functional Category: Ointment Base

DESCRIPTION

An ointment is a viscous semisolid preparation used topically on a variety of body surfaces. An ointment base is the major component of an ointment and controls its physical properties.

FUNCTIONAL MECHANISM

Ointment bases serve as vehicles for topical application of medicinal substances and also as emollients and protective agents for skin.

PHYSICAL PROPERTIES

Ointment bases are liquids with a relatively high viscosity so that solids can be suspended as a stable mixture.

Ointment bases are classified as: 1) oleaginous ointment bases that are anhydrous, do not absorb water readily, are insoluble in water, and are not removable by water (e.g., petrolatum); 2) absorption ointment bases that are anhydrous and absorb some water but are insoluble in water and are not water removable (e.g., lanolin); 3) emulsion ointment bases that are water-in-oil or oil-in-water emulsions and are hydrous, absorb water, and are insoluble in water (e.g., creams of water, oils, waxes, or paraffins); and 4) water-soluble ointment bases that are anhydrous and absorb water and are soluble in water and are water removable (e.g., polyethylene glycol).

CHEMICAL PROPERTIES

Ointment bases are selected to be inert and chemically stable.

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GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected ointment base functions: *Congealing Temperature* ⟨651⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

Functional Category: Stiffening Agent

DESCRIPTION

A stiffening agent is an agent or a mixture of agents that increases the viscosity or hardness of a preparation, especially in ointments and creams.

FUNCTIONAL MECHANISM

In general, stiffening agents are high melting point solids that increase the melting point of ointments or increase the consistency or body of creams. Stiffening agents can be either hydrophobic (e.g., hard fat or paraffin) or hydrophilic (e.g., polyethylene glycol, high molecular weight).

PHYSICAL PROPERTIES

The primary physical property relevant to stiffening agents is their high melting point or melting range. Typical melting ranges for stiffening agents range from 43° to 47° (cetyl esters wax), 53° to 57° (glyceryl distearate), 69° to 74° (glyceryl behenate), and 85° to 88° (castor oil, hydrogenated).

CHEMICAL PROPERTIES

Stiffening agents comprise a diverse group of materials that include glycerides of saturated fatty acids, solid aliphatic alcohols, esters of saturated fatty alcohols and saturated fatty acids, saturated hydrocarbons, blends of fatty alcohols and a polyoxyethylene derivative of a fatty acid ester of sorbitan, and higher ethylene glycol polymers.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected stiffening-agent functions: *Congealing Temperature* ⟨651⟩, *Melting Range or Temperature* ⟨741⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

ADDITIONAL INFORMATION

Some of the materials included as stiffening agents increase the water-holding capacity of ointments (e.g., petrolatum) or function as co-emulsifiers in creams. Examples include stearyl alcohol and cetyl alcohol.

Functional Category: Emollient

DESCRIPTION

Emollients are excipients used in topical preparations to impart lubrication, spreading ease, texture, and softening of the skin and to counter the potentially drying/irritating effect of surfactants on the skin.

FUNCTIONAL MECHANISM

Emollients help form a protective film and maintain the barrier function of the epidermis. Their efficacy may be described by three mechanisms of action: protection against the delipidizing and drying effects of surfactants, humectancy due to occlusion (by providing a layer of oil on the surface of the skin, emollients slow water loss and thus increase the moisture-retention capacity of the stratum corneum), and lubricity, adding slip or glide to the preparation.

PHYSICAL PROPERTIES

Emollients impart one or more of the following attributes to a pharmaceutical preparation: spreading capacity, pleasant feel to the touch, softness of the skin, and indirect moisturization of the skin by preventing transepidermal water loss.

CHEMICAL PROPERTIES

Emollients are either oils or are derived from components of oils as esters of fatty acids. Depending on the nature of its fatty acid ester, an emollient may be liquid, semisolid, or solid at room temperature. Generally, the higher the molecular weight of the fatty acid moiety (carbon chain length) the richer the feel and softness of the touch. Fluidity generally is imparted by shorter chain length and higher degree of unsaturation in the fatty acid moiety. The degree of branching of ester bonds also influences the emollient properties.

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GENERAL CHAPTER

The following general chapter may be useful in ensuring consistency in selected emollient functions: *Fats and Fixed Oils* ⟨401⟩.

PARENTERALS

Functional Category: Pharmaceutical Water

DESCRIPTION

Water is used as a solvent, vehicle, diluent, or filler for many drug products, especially those supplied in liquid form. These may include injectable drugs, ophthalmic drugs, inhalation solutions, and others. Water also is a vehicle for buffers and antimicrobial agents and is a volume expander for infusion solutions.

USP includes monographs for eight grades of pharmaceutical waters. Water for Injection is intended for use in the preparation of parenteral solutions. Where used for the preparation of parenteral solutions subject to final sterilization, use suitable means to minimize microbial growth, or first render the Sterile Water for Injection and, thereafter, protect it from microbial contamination. For parenteral solutions that are prepared under aseptic conditions and are not sterilized by appropriate filtration or in the final container, first render the Sterile Water for Injection and, thereafter, protect it from microbial contamination. Do not use Purified Water in preparations intended for parenteral administration. Where used for sterile dosage forms other than for parenteral administration, process the article to meet the requirements under *Sterility Tests* ⟨71⟩, or first render the Sterile Purified Water and, thereafter, protect it from microbial contamination. *USP* also contains references to other types of water, such as distilled water, deionized water, and others according to specific use as summarized in general information chapter *Water for Pharmaceutical Purposes* ⟨1231⟩.

FUNCTIONAL MECHANISM

A solvent is able to dissolve materials because it is able to disrupt the intermolecular attractive forces and to allow the individual molecules to become dispersed throughout the bulk solvent. Water is a favored solvent and vehicle in the majority of applications because it is easy to handle, safe, and inexpensive.

PHYSICAL PROPERTIES

Water is liquid at normal temperature and pressure. It forms ice at the freezing temperatures of 0° or lower, and it vaporizes at a normal boiling temperature of 100°, depending on atmospheric pressure. Vaporized water in the form of steam is used for sterilization purposes because the latent heat of steam is significantly higher than that of boiling water.

CHEMICAL PROPERTIES

Water in its pure form is neutral in pH and has very low conductivity and total organic carbon (TOC). However, pH, conductivity, and TOC are affected by storage conditions and exposure to gases in the air. Exposure to atmospheric carbon dioxide lowers water's pH. Storage in plastic containers may increase the TOC content of water over time. Water stored in glass containers may result in an increase in pH and conductivity over time.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected pharmaceutical water functions: *Injections and Implanted Drug Products* ⟨1⟩, *Bacterial Endotoxins Test* ⟨85⟩, *Total Organic Carbon* ⟨643⟩, *Water Conductivity* ⟨645⟩, *Water for Hemodialysis Applications* ⟨1230⟩, and *Water for Pharmaceutical Purposes* ⟨1231⟩.

Functional Category: Bulking Agent

DESCRIPTION

Bulking agents used in lyophilized pharmaceuticals, also referred to as freeze-dried products, include various saccharides, sugar alcohols, amino acids, and polymers. The primary function of bulking agents is to provide a pharmaceutically elegant freeze-dried cake with noncollapsible structural integrity that will reconstitute rapidly before administration. In addition, bulking agents are selected to prevent product loss caused by blow-out during freeze drying, to facilitate efficient drying, and to provide a physically and chemically stable formulation matrix. Complementary combinations of bulking agents, e.g., mannitol and a polymer, frequently are used to improve performance.

FUNCTIONAL MECHANISM

A bulking agent that readily crystallizes during lyophilization helps maintain the structural integrity of the cake formed during primary drying, thereby preventing macroscopic collapse and maintaining pharmaceutical elegance. Microscopic collapse of amorphous components in the formulation can still occur (with some potentially undesirable results) but does not result in macroscopic collapse or "meltback" if the bulking agent's properties and concentration are adequate. The bulking agent also should possess a high eutectic melting temperature with ice to permit relatively high primary drying temperatures with commensurate rapid and efficient drying and subsequent rapid reconstitution upon usage. Functional cake-forming excipients,

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such as mannitol, frequently are used because they crystallize during freezing, thereby allowing efficient drying and the formation of a structurally robust and stable cake. Amino acids and cosolvents also have been used to achieve this effect. Most biopolymer active ingredients remain amorphous upon freeze-drying, and bulking agents such as disaccharides can function as lyoprotectants by helping to maintain a stable amorphous phase during freezing and drying to prevent denaturation. Solubility enhancement of an insoluble crystalline active ingredient sometimes is achieved with the use of a biopolymer that enhances solubility or prevents crystallization during lyophilization or subsequent reconstitution. Bulking agents also are selected on the basis of biocompatibility, buffering capability, and tonicity-modifying properties.

Lyoprotectant properties of bulking agents (i.e., those that protect the drug substance during lyophilization) typically are achieved by the formation of a highly viscous glassy phase that includes the biopolymer drug substance in combination with low molecular weight amorphous saccharides such as sucrose, trehalose, or certain amino acids. A typical approach for protein pharmaceutical formulation is to combine a sugar alcohol that readily crystallizes and an amorphous diluent. This mixture acts as a lyoprotectant.

PHYSICAL PROPERTIES

Bulking agents are dissolved in aqueous solution before lyophilization. Therefore, chemical purity and the absence of bioburden and pyrogenic materials are essential properties of the excipient. However, the physical form and particle properties of the excipient generally are not relevant to the final properties of the lyophilized formulation. The solubilization process and the drying process can be facilitated by the use of volatile cosolvents such as ethanol or tertiary butyl alcohol.

The physical properties that are essential to product performance during and after lyophilization include the glass transition temperature (T_g) of the amorphous frozen concentrate before drying, the glass transition temperature of the final dried formulation cake, and the eutectic melting temperature of the crystalline bulking agent with ice. The glass transition temperature (T_g) of the formulation depends on the glass transition temperatures of the individual components, concentrations, and interactions. Although approximations can be made based on reported transition temperatures for individual components, current practice includes the measurement of formulation glass transition temperatures by thermal analysis or freeze-drying microscopy.

The physical states of the bulking agent during and after lyophilization are important physical properties. Both formulation composition and processing parameters play roles in determining whether the bulking agent is amorphous or takes a specific crystalline form. Rate of freezing, drying temperatures, and annealing are among the important process parameters used to control the physical state of the formulation and its components. Moisture retention and adsorption after lyophilization also can contribute to formulation instability and poor reconstitution.

CHEMICAL PROPERTIES

Reactivity of the bulking agent with other formulation components, especially the active ingredient, may be critical. Reducing sugars are well known to react with aromatic and aliphatic amines. Glycols may contain trace peroxide levels that can initiate oxidative degradation. The ability of saccharides and polyhydric alcohols to form hydrogen bonds to biopolymers may play a role in their lyoprotection effects.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selecting bulking agent functions: *Injections and Implanted Drug Products* ⟨1⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Thermal Analysis* ⟨891⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, and *Water–Solid Interactions in Pharmaceutical Systems* ⟨1241⟩.

Functional Category: Tonicity Agent

DESCRIPTION

To avoid crenation or hemolysis of red blood cells and to mitigate pain and discomfort if solutions are injected or introduced into the eyes and nose, solutions should be made isotonic. This requires that the effective osmotic pressure of solutions for injection must be approximately the same as that of blood. When drug products are prepared for administration to membranes, such as eyes or nasal or vaginal tissues, solutions should be made isotonic with respect to these tissues.

FUNCTIONAL MECHANISM

Tonicity is equal to the sum of the concentrations of the solutes that have the capacity to exert an osmotic force across a membrane and thus reflects overall osmolality. Tonicity applies to the impermeant solutes within a solvent—in contrast to osmolarity, which takes into account both permeant and impermeant solutes. For example, urea is a permeant solute, meaning that it can pass through the cell membrane freely and is not factored when determining the tonicity of a solution. In contrast, sodium chloride is impermeant and cannot pass through a membrane without the help of a concentration gradient and, therefore, contributes to a solution's tonicity.

PHYSICAL PROPERTIES

Solutions of sodium chloride, dextrose, and Lactated Ringer's are common examples of pharmaceutical preparations that contain tonicity agents. Not all solutes contribute to the tonicity, which in general depends only on the number of solute particles present in a solution, not the kinds of solute particles. For example, mole for mole, sodium chloride solutions display a

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higher osmotic pressure than glucose solutions of the same molar concentration. This is because when glucose dissolves, it remains one particle, but when sodium chloride dissolves, it becomes two particles: Na^+ and Cl^-. Several methods are available to calculate tonicity.

CHEMICAL PROPERTIES

Tonicity agents may be present as ionic or nonionic types. Examples of ionic tonicity agents are alkali metal or earth metal halides such as calcium chloride ($CaCl_2$), potassium bromide (KBr), potassium chloride (KCl), lithium chloride (LiCl), sodium iodide (NaI), sodium bromide (NaBr) or sodium chloride (NaCl), sodium sulfate (Na_2SO_4), or boric acid. Nonionic tonicity agents include glycerol, sorbitol, mannitol, propylene glycol, or dextrose. Sodium or potassium chloride and dextrose commonly are added to adjust tonicity.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected tonicity agent functions: *Injections and Implanted Drug Products* ⟨1⟩, *Osmolality and Osmolarity* ⟨785⟩, and *Pharmaceutical Calculations in Pharmacy Practice* ⟨1160⟩.

AEROSOLS

Functional Category: Propellant

DESCRIPTION

Propellants are compounds that are gaseous under ambient conditions. They are used in pharmaceuticals (nasal sprays and respiratory and topical formulations), cosmetics, and foods to provide force to expel contents from a container.

FUNCTIONAL MECHANISM

Propellant substances are low boiling point liquids or compressed gases that are relatively inert toward active ingredients and excipients. They can be characterized by three properties: whether they form a liquid phase at ambient temperatures and useful pressures, their solubility and/or miscibility in the rest of the formulation, and their flammability. Their performance is judged by their ability to provide adequate and predictable pressure throughout the usage life of the product.

Propellants that have both a liquid and gas phase in the product provide consistent pressures as long as there is a liquid phase present—the pressure in the headspace is maintained by the equilibrium between the two phases. In contrast, the pressure provided by propellants that have no liquid phase may change relatively rapidly as the contents of the container are expelled. As the headspace becomes larger, the pressure within the container falls proportionately. Propellants that have no liquid phase but have significant pressure-dependent solubility in the rest of the formulation have performance characteristics between those of the other two systems. In such cases, as the headspace increases the propellant comes out of solution to help to maintain the pressure of the system.

In metered-dose inhalers, the propellant has a liquid phase that is an integral part of the dispensed pharmaceutical product. Actuating the metering valve dispenses a defined volume of the liquid contents. The propellant spontaneously boils and provides atomizing and propulsive force. A predictable change in active concentration occurs from the beginning to the end of the container life cycle as the liquid phase of the propellant vaporizes to reestablish the equilibrium pressure of the system as the headspace increases.

PHYSICAL PROPERTIES

Propellants have boiling points well below ambient temperatures. A propellant's density for disperse systems and its solubility properties may be significant considerations when one selects a propellant. Apaflurane and norflurane have liquid-phase densities that are greater than that of water. Hydrocarbon propellants (butane, isobutane, and propane) and dimethyl ether have liquid-phase densities that are less than that of water.

CHEMICAL PROPERTIES

Propellants typically are stable materials. The hydrocarbon propellants (butane, isobutene, and propane) and dimethyl ether are all flammable materials. Apaflurane, carbon dioxide, nitrogen, and norflurane are nonflammable. Nitrous oxide is not flammable but supports combustion. Chlorofluorocarbon propellants are considered to be ozone-depleting substances. Their use in foods, drugs, devices, or cosmetics is regulated by 21 CFR 2.125. Albuterol metered-dose inhalers formulated with chlorofluorocarbon propellants have not been available in the United States since January 1, 2009.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected propellant functions: *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Chromatography* ⟨621⟩, and *Water Determination* ⟨921⟩.

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DRY POWDER INHALERS

Dry powder inhalers (DPIs) commonly contain few functional excipients. For example, DPIs may contain a carrier and may use a capsule shell. Other useful excipients include glidants to improve flow during manufacture of the active carrier mix. A discussion of the use of a lubricant can be found in the tablet or capsule sections above in addition to the carrier properties discussed below.

Functional Category: Carrier

DESCRIPTION

Carriers are used to help deposit the active ingredient in the lung and may have a secondary role in diluting the active to ensure that dosages can be properly metered.

FUNCTIONAL MECHANISM

The carriers are used to promote drug deposition into the lungs for better penetration or absorption in the appropriate lung location. In addition, the carrier is used to decrease the concentration of the active so the latter is adequately dosed in a uniform manner.

PHYSICAL PROPERTIES

The physical properties of carriers include appropriate morphology, hydration state, flowability, surface energy, and particle size distribution.

CHEMICAL PROPERTIES

Carriers must have suitable purity, including low microbial content and no extraneous proteins or impurities, to avoid interactions with the patient's immune system.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected carrier functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Nitrogen Determination* ⟨461⟩, *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Powder Fineness* ⟨811⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Water Determination* ⟨921⟩, *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: DPI Capsule Shells

DESCRIPTION

Capsule shells sometimes are used in DPIs. The capsule shell is used to contain the dosage amount and safeguard the inhalable powder in a DPI.

FUNCTIONAL MECHANISM

The use of capsule shell may speed pharmaceutical development because it does not require a complex device and can use premeasured drug substance or formulation. A capsule shell must not fragment into inhalable portions and should remain intact after the shell breaks to expose the powder for inhalation.

PHYSICAL PROPERTIES

Capsule shell composition generally is dictated by the drug substance's moisture content, brittleness, and electrostatic interactions with the inhalable powder.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected DPI capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Arsenic* ⟨211⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Residue on Ignition* ⟨281⟩, *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by*

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Analytical Sieving ⟨786⟩, *Uniformity of Dosage Units* ⟨905⟩, *Water Determination* ⟨921⟩, *Color—Instrumental Measurement* ⟨1061⟩, and *Water–Solid Interactions in Pharmaceutical Systems* ⟨1241⟩.

additional information

In addition to the general chapters listed above, useful information for ensuring consistency in selected capsule shell functions may be found in *Gelatin, Gel Strength (Bloom Value)*.

OPHTHALMIC PREPARATIONS

Functional Category: Antimicrobial Preservatives

DESCRIPTION

The preservative system acts as a safeguard to kill or inhibit the growth of microorganisms that may be inadvertently introduced in the product after the manufacturing process either during storage or use.

FUNCTIONAL MECHANISM

Antimicrobial preservatives work by a number of mechanisms. Quaternary ammonium compounds affect microbial cell membranes via charge interactions with phospholipids, leading to disruption of the cell membrane. Parabens also disrupt cell membrane integrity. Alcohols such as chlorbutanol and benzyl alcohol work via lipid (membrane) solvation and protein denaturation. *N*-[3-(Dimethylamino)propyl]tetradecanamide has greater antimicrobial effectiveness toward fungi and protozoa than do quaternary ammonium compounds. Similar to quaternary ammonium compounds, it disrupts plasma membrane integrity. Sorbic acid works by reduction of the sulfhydryl groups of proteins. Hypochlorite is a strong oxidizing agent. Reactions of chloramines with the amine groups of proteins can cause changes in conformation and thus loss of protein activity. Chlorine released by these reactions can react with cellular constituents, such as proteins and lipid. Polyaminopropyl biguanide accumulates in the cell membrane, blocking the entry of nutrients.

PHYSICAL PROPERTIES

To serve as an ophthalmic antimicrobial preservative, a compound should be at least sparingly soluble in water, thus providing an appreciable range of usable concentrations.

CHEMICAL PROPERTIES

A preservative must be compatible with the active and inactive ingredients of the finished product. For example, quaternary ammonium compounds are incompatible with anionic surfactants. Benzyl alcohol is incompatible with oxidizing agents. Chlorbutanol is incompatible with some nonionic surfactants. Compatibility between compounds varies with the pH of the formula. The preservative should be stable in solution at the formulation pH, usually from 5 to 8. Formulation pH can affect preservative activity by influencing how the preservative partitions between the formulation and microbes and how the preservative interacts with the target sites of the microbial cell. For example, preservatives that must pass through cell membranes before exerting activity should be formulated at a pH at which the preservative is mainly in its un-ionized state.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistent functions of selected antimicrobial preservatives: *Antimicrobial Effectiveness Testing* ⟨51⟩, *Sterility Tests* ⟨71⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, *Powder Flow* ⟨1174⟩, *Sterility Assurance* ⟨1211⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

Functional Category: Polymers for Ophthalmic Use

DESCRIPTION

Polymers are used in ophthalmic preparations to enhance the retention of active ingredients by reducing the amount of product that is lost from the eye when the patient blinks. In addition, polymers also can be components of artificial tears. Most water-soluble polymers commonly used as film-forming agents in ophthalmic preparations can be categorized as follows: 1) cellulose-based substances, 2) biologically produced gums, and 3) synthetically produced substances.

FUNCTIONAL MECHANISM

Film-forming agents for ophthalmic preparations can enhance the retention of active ingredients in the eye by a number of mechanisms. They can be used as simple viscosity-modifying agents to reduce the flow of the product, thereby slowing the rate of product loss after administration. They also can be used to form films on the surface of the eye so the drug remains deposited on the eye after the liquid portion of the product has been expelled or has evaporated. These agents can be formulated to produce a film or a gel when the product warms to body temperature (upon contacting the surface of the eye), mixing with the tear film, and/or evaporating. Some polymers have shown bio-adhesive properties on the cornea and can increase drug retention.

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PHYSICAL PROPERTIES

To serve as an ophthalmic film-forming agent, a polymer typically must be at least slightly soluble in water, thus providing an appreciable range of usable concentrations. Such polymers often increase viscosity or exhibit film- or gel-forming properties when warmed to body temperature, when exposed to the pH or solute composition and ionic strength of the tear film, or when the product evaporates.

CHEMICAL PROPERTIES

The finished product viscosity range that can be obtained with a film-forming agent is related to its chemical structure and molecular weight. Functional groups such as the pyruvate and acetate groups of xanthan gum can affect the relationship between viscosity and solution pH and ionic strength and also can determine film- and gel-forming properties. Polymer charge can influence interactions with the mucous layer of the eye. Molecular conformation, chain mobility, and degree of cross-linking also can affect the degree of swelling and thus performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistent functions of polymers for ophthalmic use: *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Particulate Matter in Ophthalmic Solutions* ⟨789⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, and *Powder Flow* ⟨1174⟩. In addition, the general chapters listed under *Functional Category: Film-Forming Agents* also may be appropriate for the evaluation of polymers for ophthalmic use.

TRANSDERMALS AND PATCHES

Functional Category: Adhesive

DESCRIPTION

Topical drug delivery systems (e.g., transdermals or skin patches) require the use of adhesives to maintain contact between the applied drug delivery system and the skin. Adhesives can be intercalated as a separate layer between the formulation matrix and the skin surface, incorporated as a part of the formulation matrix itself, or applied to the periphery of the topical delivery system.

FUNCTIONAL MECHANISM

Adhesion is the tendency of dissimilar surfaces to adhere to one another as a result of one or more types of interactions. For topical drug delivery systems, these adhesive interactions generally are chemical (primarily electrostatic) or dispersive (van der Waals and/or hydrogen bonding) in nature, although there is the possibility of mechanical interaction via the interlocking of microscopic asperities.

PHYSICAL PROPERTIES

In general, the adhesives used in transdermals or skin patches are pressure-sensitive materials whose performance is best characterized by physical test methods for tackiness and viscoelasticity of the adhesive per se and viscosity of a solution of the adhesive.

CHEMICAL PROPERTIES

In transdermals, the most widely used pressure-sensitive adhesives are acrylic, rubber, and silicone polymers. Acrylic polymer adhesives include various esters of acrylic or methacrylic acid, acrylamide, methacrylamide, *N*-alkoxyalkyl, or *N*-alkyl-acrylamides. Polyisobutylenes and polysiloxanes are among the most common rubber-based and silicone-based adhesives, respectively. The molecular weight and compositional distribution of the polymers are critical to the replication of the adhesive's efficacy from batch to batch.

GENERAL CHAPTERS

The following general chapters may be useful in evaluating the suitability of adhesives used in transdermals: *Tensile Strength* ⟨881⟩ and *Viscosity—Capillary Methods* ⟨911⟩.

Functional Category: Film-Forming Agent

DESCRIPTION

Film-forming agents used as the formulation matrix of topical drug delivery systems (e.g., transdermals or skin patches) or in conjunction with such systems comprise a flexible, nontacky but adherent film, in whole or in part, applied to the skin surface.

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FUNCTIONAL MECHANISM

Film formation results from the progressive loss of solvent (or dispersion medium) from a solution (or dispersion) of a film-forming agent, whether in particulate or molecularly dispersed form. Solvent (or dispersion medium) loss leads to closer molecular or particulate packing and increased interaction among the film-forming agent molecules or particles. Ultimately, a continuous film is formed as a result of increased molecular entanglement or particulate sintering.

PHYSICAL PROPERTIES

Properties critical to successful film formation include the film-forming agent's glass transition temperature (T_g), the viscosity of the solution or dispersion, and the surface characteristics of the substrate. Viscoelastic properties such as elastic modulus, viscous modulus, and intrinsic or complex viscosity describe functional characteristics, such as adhesion, for a pressure-sensitive adhesive component. Adhesion to a substrate and tack and shear tests can be used for batch release.

CHEMICAL PROPERTIES

Typical film-forming agents are thermoplastic or thermosetting high molecular weight polymers or copolymers, often in the form of aqueous dispersions or latex compositions. Cellulosic polymers, vinyl polymers and copolymers, and acrylic and methacrylic acid polymers and copolymers frequently are used in topical delivery systems as film-forming agents.

GENERAL CHAPTERS

The following general chapters may be useful in evaluating the suitability of film-forming agents used in transdermals and patches: *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

RADIOPHARMACEUTICALS

Radiopharmaceuticals commonly contain categories of excipients that also are used in conventional drugs. For example, radiopharmaceutical capsules may contain diluents and necessarily use a capsule shell, and parenteral radiopharmaceuticals may contain pharmaceutical water, diluents, tonicity agents, pH modifiers, antimicrobial preservatives, chelating and/or complexing agents, and antioxidants. Many radiopharmaceuticals differ from conventional drugs, however, because their preparation (reconstitution) involves one or more chemical reactions that require unusual excipients. Furthermore, the self-absorption of emitted radiation may result in the radiolytic decomposition of many radiopharmaceuticals. Hence, several excipients are used predominately in radiopharmaceutical formulations, although they occasionally may be used for other drugs.

Functional Category: Reducing Agent

DESCRIPTION

Reducing agents generally are required for technetium Tc 99m radiopharmaceuticals. Technetium Tc 99m, in the chemical form of sodium pertechnetate (+7 oxidation state), must be reduced to a lower oxidation state so that it can be chelated or otherwise complexed by the intended ligand to form the final Tc 99m radiopharmaceutical. The reducing agent, typically a stannous salt, generally is formulated in the kit for the preparation of the technetium Tc 99m radiopharmaceutical.

FUNCTIONAL MECHANISM

The reducing agent (e.g., stannous ion) must be present in sufficient quantity to reduce all of the technetium atoms to the intended oxidation state but must not produce undesired reduction products or other impurities (e.g., stannous hydroxide precipitates).

PHYSICAL PROPERTIES

Reducing agents (e.g., stannous salts) must be readily soluble in water.

CHEMICAL PROPERTIES

Reducing agents (e.g., stannous salts) are sensitive to oxidation by atmospheric oxygen and oxidizing species in solution. Hence, lyophilized contents of kit vials must be filled with a nonoxidative gas such as nitrogen or argon. The reducing agent also must be stable at the intended pH of the formulated product.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected reducing agent functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

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Functional Category: Transfer Ligand

DESCRIPTION

In the preparation of certain radiopharmaceuticals, the radiometal (e.g., stannous-reduced technetium Tc 99m) is first chelated by a relatively weak chelating ligand and then is transferred to the principal chelating ligand or complexing moiety. Examples of such transfer ligands include citrate, gluconate, and tartrate.

FUNCTIONAL MECHANISM

Transfer ligands typically undergo rapid reactions with reduced technetium to form weak chelates, thus keeping the reduced technetium in a soluble form until it is transferred to the principal ligand. This procedure is especially useful when the kinetics of complexation with the principal ligand is slow or when a heating step is necessary to expose chelating groups on the principal ligand.

PHYSICAL PROPERTIES

Transfer ligands must be readily soluble in water.

CHEMICAL PROPERTIES

Transfer ligands must have rapid complexation kinetics and must form relatively weak chelates compared to complexation with the principal ligand.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected transfer ligand functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

Functional Category: Colloid Stabilizing Agent

DESCRIPTION

Lyophobic colloids tend to clump together and form large aggregates to minimize their surface-area-to-volume ratio. Colloid stabilizing agents are relatively large lyophilic molecules that coat the surface of each individual colloid particle and prevent or inhibit clumping. Examples of colloid stabilizing agents include gelatin and dextran.

FUNCTIONAL MECHANISM

The colloid stabilizing agent coats the surface of the lyophobic colloid particles, making them appear lyophilic. Additionally, the colloid stabilizing agent may be charged, thus causing the coated colloid particles to repel one another.

PHYSICAL PROPERTIES

Colloid stabilizing agents must be readily soluble in water.

CHEMICAL PROPERTIES

Colloid stabilizing agents must be capable of coating the lyophobic colloid particles, e.g., by electrostatic attraction of an opposite charge.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected colloid stabilizing agent functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

Functional Category: Free Radical Scavenger

DESCRIPTION

Radiation interactions with water and other molecules frequently produce free radicals. Free radical scavengers preferentially interact with oxidative or reductive free radicals that otherwise would result in degradation of formulation components. In the case of radiopharmaceuticals, free radical scavengers can be used to enhance radiochemical purity. Examples of free radical scavengers include methylene blue and aminobenzoic acid.

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FUNCTIONAL MECHANISM

Free radical scavengers preferentially interact with radiolytically produced free radicals before these free radicals can interact with the radiopharmaceutical and produce radiochemical impurities.

PHYSICAL PROPERTIES

Free radical scavengers must be readily soluble in water.

CHEMICAL PROPERTIES

Free radical scavengers must be capable of preferentially interacting with free radicals without causing other effects.

⟨1061⟩ COLOR—INSTRUMENTAL MEASUREMENT

The observed color (see ⟨631⟩ *Color and Achromicity*) of an object depends on the spectral energy of the illumination, the absorbing characteristics of the object, and the visual sensitivity of the observer over the visible range. Similarly, it is essential that any instrumental method that is widely applicable take these same factors into account.

Instrumental methods for measurement of color provide more objective data than the subjective viewing of colors by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate and precise measurements of color and color differences that do not drift with time. The basis of any instrumental measurement of color is that the human eye has been shown to detect color via three "receptors." Hence, all colors can be broken down into a mixture of three radiant stimuli that are suitably chosen to excite all three receptors in the eye. Although no single set of real light sources can be used to match all colors (i.e., for any three lights chosen, some colors require a negative amount of one or more of the lights), three arbitrary stimuli have been defined, with which it is possible to define all real colors. Through extensive color-matching experiments with human subjects having normal color vision, distributing coefficients have been measured for each visible wavelength (400 nm to 700 nm) giving the relative amount of stimulation of each receptor caused by light of that wavelength. These distribution coefficients $\bar{x}$, $\bar{y}$, $\bar{z}$, are shown below. Similarly, for any color the amount of stimulation of each receptor in the eye is defined by the set of *Tristimulus values* (X, Y, and Z) for that color.

The relationships between the distribution coefficient (see *accompanying figure*) and the tristimulus values are given in the equations

$$X = \int_0^\infty f_\lambda \bar{x}_\lambda P_\lambda d\lambda / Y',$$

$$Y = \int_0^\infty f_\lambda \bar{y}_\lambda P_\lambda d\lambda / Y', \text{and}$$

$$Z = \int_0^\infty f_\lambda \bar{z}_\lambda P_\lambda d\lambda / Y',$$

in which

$$Y' = \int_0^\infty \bar{y}_\lambda P_\lambda d\lambda, P_\lambda$$

is the spectral power of the illuminant, and f_λ is either the spectral reflectance (ρ_λ) or spectral transmittance (τ_λ) of the material.

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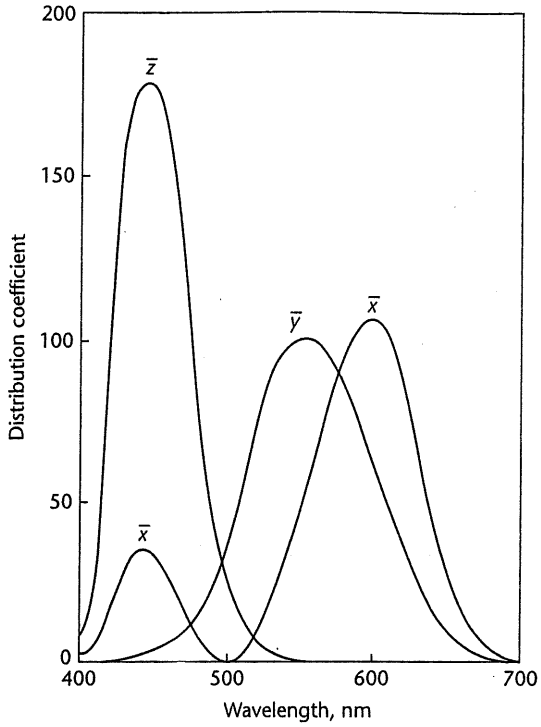

Distribution Coefficients from 400 to 700 nm

Once the tristimulus values of a color have been determined, they may be used to calculate the coordinates of the color in an idealized three-dimensional color space referred to as a *visually uniform color space.* Many sets of color equations have been developed in an attempt to define such a space. The equations given in this chapter represent a compromise between simplicity of calculation and conformance with ideality.

The coordinates of a color in a visually uniform color space may be used to calculate the deviation of a color from a chosen reference point. Where the instrumental method is used to determine the result of a test requiring color comparison of a test preparation with that of a standard or matching fluid, the parameter to be compared is the difference, in visually uniform color space, between the color of the blank and the color of the test specimen or standard.

PROCEDURE

The considerations discussed under *Ultraviolet-Visible Spectroscopy* ⟨857⟩ apply to instrumental color measurement as well. In the spectrophotometric method, reflectance or transmittance values are obtained at discrete wavelengths throughout the visible spectrum, a band width of 10 nm or less being used. These values are then used to calculate the tristimulus values through the use of weighting factors.[1] In the colorimetric method, the weighting is performed through the use of filters.

In the measurement of the spectral reflectance of opaque solids, the angle of viewing is separated from the angle of illumination in such a manner that only rays reflected diffusely from the test specimen enter the receptor. Specular reflection and stray light are excluded.

For the measurement of the spectral transmittance of clear liquids, the specimen is irradiated from within 5 degrees of the normal to its surface, and the transmitted energy measured is that confined within 5 degrees from the normal. The color of solutions changes with the thickness of the layer measured. Unless special considerations dictate otherwise, a layer 1 cm thick should be used.

The methods described here are not applicable to hazy liquids or translucent solids.

Calibration

For purposes of calibration, one of the following reference materials may be used, as required by instrument geometry. For transmittance measurements, purified water may be used as a white standard and assigned a transmittance of 1.000 at all wavelengths. Then the tristimulus values X, Y, and Z for CIE source C are 98.0, 100.0, and 118.1, respectively. For reflectance measurements, opaque porcelain plaques, whose calibration base is the perfect diffuse reflector and whose reflectance

[1] Typical weighting factors are given by ASTM Z58.7.1-1951 as reported in the *Journal of the Optical Society of America,* Vol. 41, 1951, pages 431-439.

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characteristics have been determined for the appropriate instrumental geometry, may be used.[2] If the geometry of sample presentation precludes the use of such plaques, pressed barium sulfate, white reflectance standard grade, may be used.[3]

After calibration with the above-mentioned materials, it is desirable whenever possible to measure a reference material as close to the color of the sample as possible. If a sample of the material being tested is not suitable for use as a long-term standard, color chips are available[4] which span the entire visually uniform color space in small increments. The use of such a reference standard is encouraged as a means of monitoring instrument performance even for absolute color determinations.

Spectrophotometric Method

Determine the reflectance or transmittance from 380 to 770 nm at intervals of 10 nm. Express the result as a percentage, the maximum being 100.0. Calculate the tristimulus values X, Y, and Z as follows.

REFLECTING MATERIALS

For reflecting materials the quantities X, Y, and Z are

$$X = \sum_{380}^{770} \rho_\lambda \bar{x}_\lambda P_\lambda \Delta\lambda / Y',$$

$$Y = \sum_{380}^{770} \rho_\lambda \bar{y}_\lambda P_\lambda \Delta\lambda / Y', \text{and}$$

$$Z = \sum_{380}^{770} \rho_\lambda \bar{z}_\lambda P_\lambda \Delta\lambda / Y',$$

in which

$$Y' = \sum_{380}^{770} \bar{y}_\lambda P_\lambda \Delta\lambda \rho_\lambda$$

is the spectral reflectance of the material, $\bar{x}_\lambda P_\lambda$, $\bar{y}_\lambda P_\lambda$, and $\bar{z}_\lambda P_\lambda$ are known values associated with each Standard Source,[1,2] and $\Delta\lambda$ is expressed in nm.

TRANSMITTING MATERIALS

For transmitting materials, the quantities X, Y, and Z are calculated as above, τ_λ (spectral transmittance) being substituted for ρ_λ.

Colorimetric Method

Operate a suitable colorimeter[5] to obtain values equivalent to the tristimulus values, X, Y, and Z. The accuracy with which the results obtained from the filter colorimeter match the tristimulus values may be indicated by determining the tristimulus values of plaques of strongly saturated colors and comparing these values with those computed from spectral measurements on a spectrophotometer.

INTERPRETATION

Color Coordinates

The Color Coordinates, L*, a*, and b* are defined by

$$L^* = 116(Y/Y_0)^{1/3} - 16,$$

$$a^* = 500[(X/X_0)^{1/3} - (Y/Y_0)^{1/3}], \text{ and}$$

$$b^* = 200[(Y/Y_0)^{1/3} - (Z/Z_0)^{1/3}]$$

in which X_0, Y_0, and Z_0 are the tristimulus values of the nominally white or colorless standard, and $Y/Y_0 > 0.01$. Usually they are equal to the tristimulus values of the standard illuminant, with Y_0 set equal to 100.0. In this case $X_0 = 98.0$ and $Z_0 = 118.1$.

[2] Suitable items are available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.
[3] Suitable material is available from Eastman Kodak Company, Rochester, NY 14650, as "White Reflectance Standard."
[4] Centroid Color Charts may be obtained from suppliers of instruments for measurement of color.
[5] A suitable tristimulus colorimeter is available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

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Color Difference

The total Color Difference ΔE* is

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

in which ΔL*, Δa*, and Δb* are the differences in color coordinates of the specimens being compared.

Instrumental variables can influence results. Although reliable comparisons can be made between similar colors measured concomitantly, results obtained on different instruments or under different operating conditions should be compared with caution. If it is necessary to compare data obtained from different instruments or taken at different times, etc., it is very helpful to have concomitant data obtained on a standard reference material such as color chips for opaque materials. Comparison of the readings on the reference material helps to identify variations caused by instrument performance.

⟨1062⟩ TABLET COMPRESSION CHARACTERIZATION

1. BACKGROUND
2. COMPRESSION PHASES
3. TABLET COMPRESSION CHARACTERIZATION EQUIPMENT
    3.1 Hydraulic Press
    3.2 Instrumented Research Tablet Press
    3.3 Tablet Press Emulator
    3.4 Compaction Simulator
    3.5 Instrumented Production Tablet Press
4. TOOLING
5. PUNCH DISPLACEMENT–TIME PROFILES
6. TABLET MECHANICAL STRENGTH
7. TABLET POROSITY AND SOLID FRACTION
8. MANUFACTURABILITY PROFILE
9. TABLETABILITY PROFILE
10. COMPRESSIBILITY PROFILE
11. COMPACTIBILITY PROFILE
12. TABLET COMPRESSION PROFILE
13. MACHINE SPEED SENSITIVITY
14. CONCLUSIONS AND RECOMMENDATIONS
GLOSSARY
REFERENCES

1. BACKGROUND

The tablet is currently the most widely used dosage form for oral drug delivery (see *Pharmaceutical Dosage Forms* ⟨1151⟩). The advantages of the tablet include economy of manufacture, patient convenience, and compliance. Tablets may also offer additional advantages over other dosage forms, such as superior physical or chemical stability.

Powder compression is a critical process in manufacturing the tablet dosage form. Although this process has been used routinely for over a century, problems related to powder compression in pharmaceutical formulation development and manufacturing persist. Common problems include tablet failures, such as capping and lamination (*1–3*), high friability, powder sticking to punch surfaces or the die wall, and insufficient mechanical strength to withstand stress in downstream processing. Some formulations may exhibit acceptable compression characteristics during early development where production volumes are usually low but become problematic during scale-up.

The properties of compressed tablets are sensitive to both material characteristics and process parameters. The characteristics of the equipment used and the ambient conditions of temperature and humidity are also key factors that influence tablet compression. Physical properties such as particle size (*4*), particle shape (*5,6*), surface texture (*7*), crystallinity, and moisture content (*8*) influence powder tableting performance by affecting the bonding strength and/or the bonding area (*9,10*). The powdered material mechanical properties, such as particle hardness, elastic properties, viscoelastic properties, plasticity, and particle brittleness, also affect tablet strength (*11–13*). The extent of lubrication is yet another important factor that affects powder compression. A lubricant is typically added to the drug product blend formulation as a final step prior to compression to reduce frictional forces during compression and tablet ejection. This added lubricant, however, may adversely impact tablet mechanical strength and dissolution release rate.

This chapter describes the current understanding of this specialized area and outlines experimental methodologies for characterization of tablet compression to provide guidance for standardized compression test procedures and use of terminology. Although the fundamental concepts described here are also applicable to other processes, such as plug formation during encapsulation and roller compaction, the focus of this article is on tableting.

[NOTE—The *Glossary* defines terms with the International System of Units (SI), commonly used in pharmaceutical tablet compression.]

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2. COMPRESSION PHASES

Powder compression behavior is governed by the physical and mechanical properties of the material as well as aspects of the compression process such as pressure (i.e., stress), degree of deformation (i.e., strain), and rate of deformation (i.e., strain rate). Therefore, knowledge of stress, strain, and strain rate is important for understanding powder behavior during the compression process. The majority of pharmaceutical tablets are manufactured by "uniaxial powder compression". That is, each tablet is formed by the densification of a loosely packed powder sample confined within a rigid die using two rigid punches that approach from above and below (in a vertical plane). This tablet formation process is often described as occurring in four stages, as shown in *Figure 1*:

1. Particle rearrangement
2. Compression
3. Decompression
4. Ejection

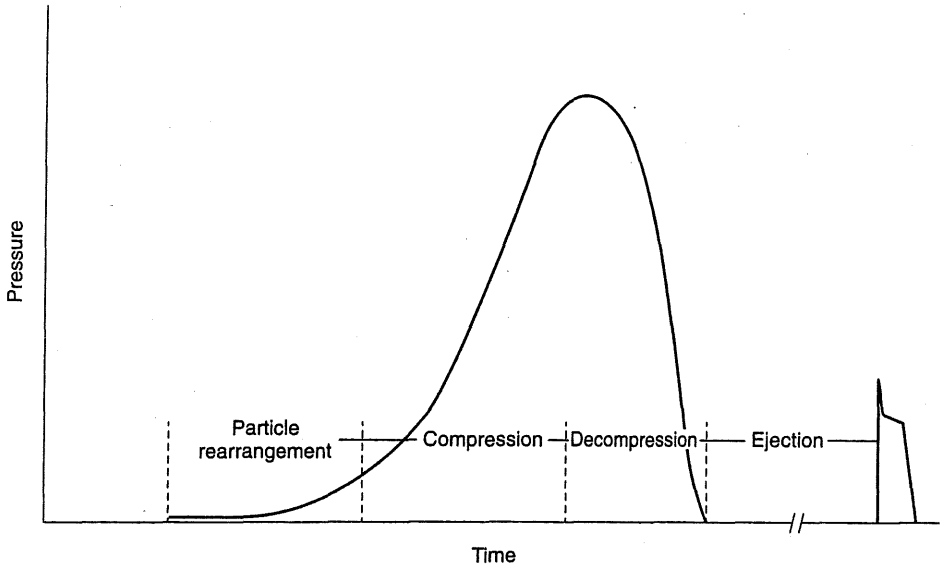

Figure 1. Stages of powder compression (lower punch pressure).

In the first stage, *Particle rearrangement*, particles typically change positions through slippage, rotation, or translational movement, thereby reducing or eliminating pores in the powder without significant irreversible deformation. At the end of the first stage, where the particles have reached their fullest extent of rearrangement, the powder is usually significantly denser than the starting powder because of reduced pore volume. Any further reduction in compact volume would require particle deformation.

During the second stage, *Compression*, particles are deformed at points of contact with other particles, the die wall, or the punch surfaces. During this stage, compression pressure often increases rapidly, causing volume reduction as the powder density increases. Under pressure, particles initially undergo elastic deformation. Depending on mechanical properties and stress at points of contact, particles can subsequently undergo varying degrees of fragmentation and/or plastic deformation. If fragmentation takes place early in the second stage, some of the fragments may undergo further rearrangement. However, relative particle movement is limited when the powder is highly consolidated. For most pharmaceutical materials, plastic deformation is an important part of the compression process that leads to an increase in the area of contact between particles, contributing to higher compact strength. Clean particle surfaces generated from fragmentation also contribute to higher compact strength. The deformation behavior and resultant tablet mechanical properties of many pharmaceutical powders are also sensitive to the compression speed (punch velocity) and the length of time at which the powder is held under pressure at a constant volume (dwell time). Tablet density and tensile strength of speed-sensitive powders (such as starch) depend on tableting speed, with higher speeds (i.e., shorter dwell times) generally producing less-dense tablets with lower strength. The end of the second stage is usually the time of highest compression pressure.

During the third stage, *Decompression*, the punches retract, resulting in a decreasing axial punch pressure. As axial pressure is reduced to zero during decompression, residual die wall pressure typically exists in the radial direction. During this phase, particles primarily undergo elastic recovery, depending on both the pressure and the mechanical properties of the particles. Elastic recovery may provide insight into the elastic deformation that the powder experienced during compression. Excessive elastic recovery may reduce the interparticle bonding and can result in a significant decrease in tablet mechanical strength. These same three stages apply to a pre-compression step in tablet manufacturing, which often involves a lower compression pressure and may be added as a precursor to the main compression step.

During the fourth stage, *Ejection*, the tablet is typically pushed out of the die by the lower punch. As the tablet emerges from the die, the ejected portion of the tablet is free to expand radially due to elastic recovery (i.e., release of residual die wall pressure). Significant shear stress may develop within the tablet and at the edges of the tablet–die interface because the lower portion of

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the tablet remains constrained by the die wall. In severe cases, this shear stress can result in tablet lamination or capping. The formation of a dense and defect-free compact depends on the ability of the particles to form interparticulate bonds during compression, and the ability of these bonds to withstand elastic expansion during the decompression and ejection phases. Tooling shape and size may also affect the properties of the compressed tablet because they affect density and stress distribution during compression. The thermodynamics of the compression process, e.g., via compression calorimetry (*14,15*), is another important aspect of powder compression, and it can be studied using instrumented presses (see *3. Tablet Compression Characterization Equipment*).

3. TABLET COMPRESSION CHARACTERIZATION EQUIPMENT

Various compression methods have been used to characterize the compression properties of pharmaceutical powders. Each method has benefits and limitations. To characterize powder-compression properties, a compression pressure (stress) is applied to a powder to produce a coherent, compacted specimen or tablet. The loading system used to apply the compression pressure may have several designs. Typical compression instruments used in the pharmaceutical industry include hydraulic presses, instrumented research tablet presses, tablet press emulators, compaction simulators, and instrumented production tablet presses.

3.1 Hydraulic Press

When a hydraulic press is used, typically only the peak hydraulic pressure value is easily accessible. Generally, compression and decompression speeds are not precisely controlled, and these processes occur over a period of seconds or more. Tablets produced on such devices may then be tested for strength and density, as well as other performance properties such as friability, disintegration, or dissolution. Such devices provide compression data for comparing materials and formulations but are not necessarily predictive of tableting performance during high-speed tableting.

3.2 Instrumented Research Tablet Press

Research tablet presses have two basic designs: eccentric single-station and rotary multi-station presses. Eccentric single-station presses typically are mechanically driven and compress the tablet using the upper punch with a sinusoidal position profile; the lower punch is typically fixed during the compression cycle. Research rotary multi-station tablet presses are scaled-down versions of production-scale presses, and they compress tablets via the movement of both the upper and lower punch as they pass under a pair of compression rolls. The geometry of the punch head (i.e., curvature and flat area) also influences the shape of the punch position profile on a rotary tablet press.

3.3 Tablet Press Emulator

Tablet press emulators are similar to rotary tablet presses in that they use compression rolls to move the punches together to form the tablet, and therefore punch displacement–time profiles (see *5. Punch Displacement–Time Profiles*) are highly representative of rotary machines. However, the tablet press cycle time is longer than that of rotary presses because the compression track is linear.

3.4 Compaction Simulator

Compaction simulators are single-station presses that utilize hydraulic or mechanical power to move the punches and are designed to match the displacement profile of a given high-speed press by means of a computer. Compaction simulators can also be programmed to simulate any punch position profile, which is useful for fundamental material characterization.

3.5 Instrumented Production Tablet Press

Instrumented production tablet presses also use a compression roll design to form a tablet. A large number of compression stations enables greater production throughput and/or multi-layer tablet or tablet-in-tablet capability.

4. TOOLING

Tablets can be manufactured with a variety of tooling sizes and shapes. Commonly used tooling shapes for basic material characterization are flat-faced, flat-faced with beveled edge, and standard round concave tooling that produces convex-shaped tablets. A wide variety of tooling shapes may be used in the production of pharmaceutical products, based on technical and commercial considerations (*16*). Tooling may be embossed (i.e., raised markings on punch surface), thus introducing debossed markings on the compressed tablets. Depending on the powder compression properties and manufacturing process parameters, the tablet tooling design may affect the tablet mechanical integrity.

5. PUNCH DISPLACEMENT–TIME PROFILES

Tablet compression equipment can be used to produce a variety of punch displacement–time profiles. *Figure 2* is an illustration of three of the simplest profiles used for fundamental material characterization.

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The properties of a compressed powder are dependent upon several factors, including compression pressure, compression speed, and the compression profile. Compression experiments may be performed with one moving punch and one stationary punch; one moving upper punch and a fixed base (i.e., no lower punch); or two independently moving upper and lower punches (double-sided compression). Because the compression set-up and parameters can affect measured compact properties, it is important to specify the experimental details when reporting results. Typical compression profiles include:

- Linear compression and decompression phases that yield saw-tooth punch displacement–time profiles (*Figure 2A*)
- Square punch displacement–time profiles (*Figure 2B*)
- Modified sinusoidal punch displacement–time profiles typical of a rotary tablet press (*Figure 2C*)

Either one punch (single-sided compression) or both punches (double-sided compression) may follow these profiles. Accurate measurements of the displacement–time profiles, as well as the resultant forces applied, require high-accuracy instrumentation, and in some cases correction for system deformation (i.e., elastic deformation of punches and other machine components). Punch displacement–time profiles for most production-scale tableting machines are a combination of the sinusoidal and square profiles, where the compression and decompression phases follow the sinusoidal profile and between them there is a flat portion representing the dwell time.

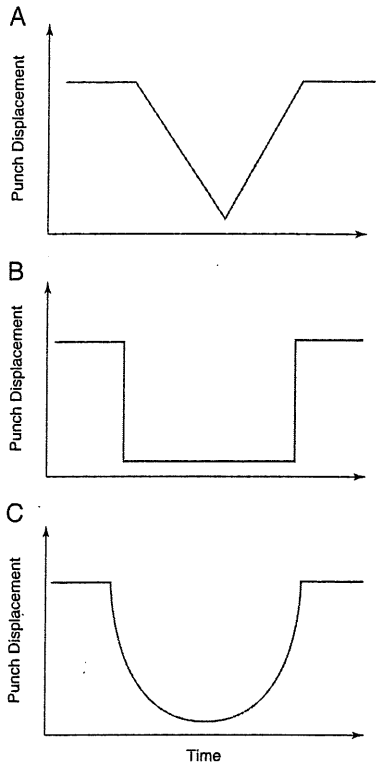

Figure 2. Punch displacement–time profiles: A) saw-tooth profile, B) square profile, and C) modified sinusoidal profile. Only one curve for punch displacement over time is represented (e.g., upper-punch movement).

6. TABLET MECHANICAL STRENGTH

Tablet strength is primarily influenced by the particle–particle bond strength and true areas of contact (e.g., surface area over which attractive force between particles is significant) (*17*). When particle surfaces are brought into close proximity, interparticle interactions (e.g., van der Waals forces) are maximized and typically lead to strong interparticle bonding. Tablet mechanical strength can be quantified by measuring the maximum stress, either compressive or tensile, that a tablet can sustain prior to failure (breaking). A commonly used test is to place tablets between two platens and measure the force necessary to fracture the tablets; this test is described in *Tablet Breaking Force* ⟨1217⟩. For conventional round tablets with a circular cross-section, loading occurs across the diameter of the tablet and is sometimes referred to as "diametral loading". Other methods, such as three-point or four-point bending tests, are also available but are less frequently used in production settings because they can require more complex equipment and analysis. Tablet strength, as determined by these tests, is often referred to in the pharmaceutical industry as "hardness", although a more exact term is "breaking force". In material science, hardness refers to the resistance of a surface to penetration or indentation (e.g., Mohs hardness, indentation hardness, or permanent deformation pressure). The tablet breaking force value serves as both a criterion by which to guide product development and a quality control specification.

Tablet strength can be affected by several factors including:

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- Tablet size and shape: Because breaking force is affected by tablet size and shape, a more reliable parameter for quantifying mechanical strength of a tablet is tensile strength. For cylindrical or convex tablets with simple shapes, tensile strength may be calculated from the diametral test, described in ⟨1217⟩.
- Relative density: Tablet strength increases as powders are compressed to a higher relative density.
- Time and storage conditions: Tablets can relax or be influenced by environmental conditions (e.g., relative humidity), therefore tablet storage conditions and length of storage time before testing should be specified for reproducible strength determinations.
- Formulation composition and manufacturing process: Each component has unique mechanical properties, and in some cases these properties can impact the mechanical properties of other components. For example, incorporation of a lubricant, which is meant to reduce adherence to manufacturing equipment, can reduce interparticulate bonds. Problems in manufacturing processes, such as over-mixing or over-granulation, also can influence tablet strength.

Tablet performance attributes, such as disintegration, dissolution, and friability, may be affected by tablet compression and may also be reflected in the tablet mechanical strength. Typically, a tablet of lower strength will have faster disintegration and dissolution as well as higher friability (see *Tablet Friability* ⟨1216⟩).

7. TABLET POROSITY AND SOLID FRACTION

Virtually all pharmaceutical compacts contain porous regions (pores). Tablet porosity is a measure of the volume of the tablet that consists of pores, or "void space". It is critically important to consider tablet porosity when quantitatively characterizing tableting properties, because it has a substantial effect on measured compact properties. Tablet solid fraction, also referred to as relative density, is a measure of the volume of solid material in a compact and may be calculated using *Equation 1*. Tablet solid fraction and porosity are related, as shown in *Equation 2*. The true density of a material is the average mass/unit volume (e.g., g/cm^3) exclusive of all voids (see *Density of Solids* ⟨699⟩). Typical true densities of organic powders of pharmaceutical interest are in the range of 1.0–1.7 g/cm^3, whereas inorganic ingredients may be in the range of 2.0–3.0 g/cm^3 (*18*,*19*).

$$\text{Solid Fraction} = \frac{\text{(density of the tablet)}}{\text{(true density of material)}} = \frac{\dfrac{\text{(mass of tablet)}}{\text{(volume of tablet)}}}{\text{(true density of material)}} \qquad (1)$$

$$\text{Porosity} = 1 - \text{Solid fraction} \qquad (2)$$

Simple tablet geometries (e.g., round flat-faced) are often used for research purposes, to simplify the determination of tablet volume by using the measurement of tablet dimensions. For more complex tablet shapes, alternative methods of determining tablet volume, such as the use of instruments that quantify envelope volume, may be used. Typical pharmaceutical tablets have porosities between 0.1 and 0.4, depending on the material properties and the conditions used to produce the tablet (*20*). A porosity of 0 would correspond to a theoretical tablet mass in which all pores had been eliminated, resulting in a compact consisting entirely of solid material (i.e., solid fraction = 1). With increasing compression pressure, pores are eliminated through particle rearrangement and deformation, and the tablet porosity decreases unless extensive elastic recovery of the tablet after decompression causes cracks or other defects in the tablet.

8. MANUFACTURABILITY PROFILE

Tablet breaking force is often measured as a function of compression force. A plot of breaking force versus compression force is useful for monitoring changes in the tableting behavior of a powder with a fixed tablet size, shape, and weight produced under similar compression conditions (i.e., production speed and force), such as those obtained using a specific rotary tablet press. In this case, the relationship between tablet breaking force and compression force may be termed "manufacturability", because it is often the criterion used in a production setting to monitor tablet compression. See *Figure 3* for an example of a manufacturability profile.

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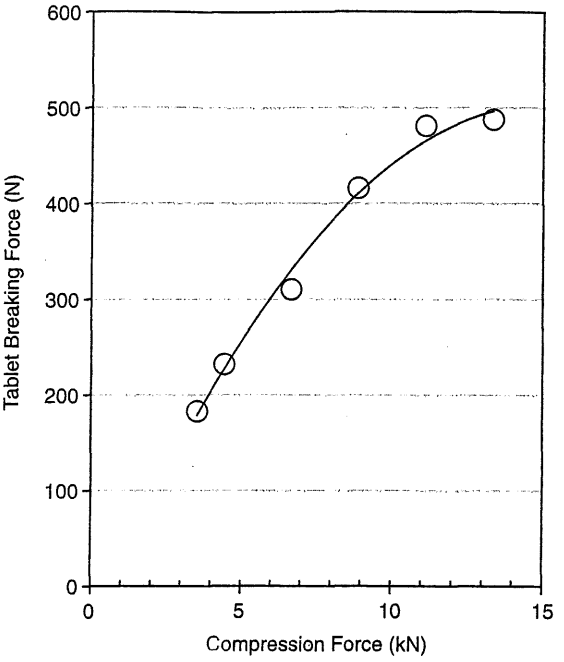

Figure 3. Example of a manufacturability profile.

9. TABLETABILITY PROFILE

Although valuable in a manufacturing setting, tablet breaking force should be replaced with tablet tensile strength for quantifying tablet mechanical strength (see ⟨1217⟩), and compression force should be replaced with compression pressure (compression force per unit area of the punch tip cross-section). These changes will minimize the impact of tablet size, thickness, and weight on compression data analysis. The relationship between tablet tensile strength and compression pressure is termed "tabletability". Tablet tensile strength usually increases initially with increasing compression pressure. Depending on tablet composition, tensile strength can either continue to increase or gradually level off at higher pressures. It is also possible that the tablet tensile strength may decrease with increasing pressure, a phenomenon known as overcompression. This decrease in tablet strength with increasing pressure is most often the result of tablet defects of some materials that occur at higher compression pressures. Because of the diversity of powder tabletability behaviors, it is beneficial to determine the tensile strength of tablets prepared under a range of compression pressures, instead of a single pressure, if possible. This will help obtain an accurate characterization of powder tabletability. When the available resources or materials are limited, the compression pressure required to make a compact at a specified tensile strength (e.g., 1 MPa) can be used to compare compression properties of different powders. The tabletability of pharmaceutical materials can often be described by *Equation 3* for a typical range of compression pressures, where K and B are empirical constants:

$$\log(\textit{tensile strength}) = K \log(\textit{compression pressure}) + B \quad (3)$$

See *Figure 4* for an example of a tabletability profile.

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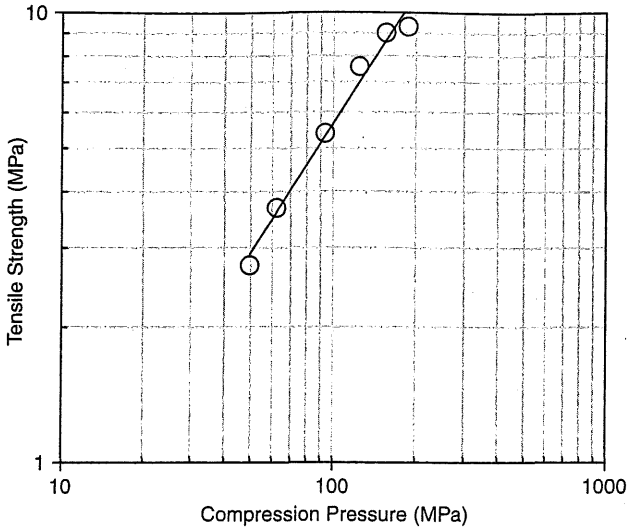

Figure 4. Example of a tabletability profile.

10. COMPRESSIBILITY PROFILE

Compressibility is the dependence of tablet solid fraction (or porosity) on compression pressure. The compressibility curve can be obtained by plotting tablet solid fraction as a function of compression pressure. See *Figure 5* for an example of a compressibility profile.

For many pharmaceutical materials, *Equation 4* can be used to describe compressibility over a typical range of tablet solid fractions, where *a* and *b* are empirical constants:

$$\log(\textit{compression pressure}) = a \times (\textit{solid fraction}) + b \quad (4)$$

The compression pressure necessary to form a compact with a specified solid fraction (e.g., 0.85) may be used to compare diverse pharmaceutical materials. Use of 0.85 as a reference solid fraction is convenient because many, although not all, pharmaceutical powders can be compressed to this solid fraction, and a reference solid fraction enables comparative assessments of tablet property measurements. Alternative values for the reference solid fraction may be used as needed.

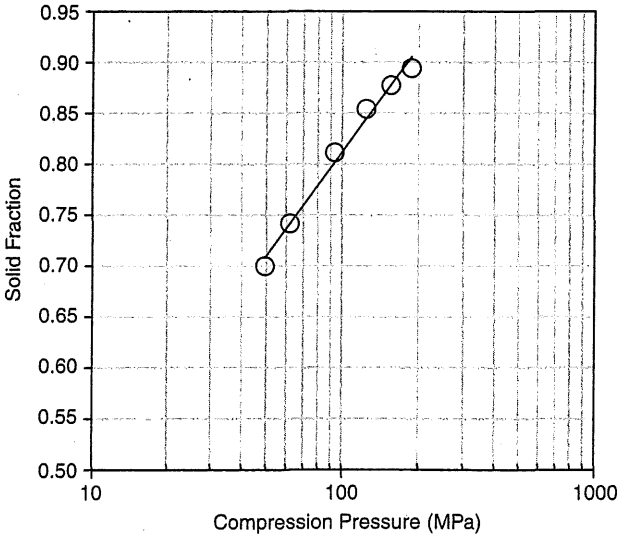

Figure 5. Example of a compressibility profile.

Compressibility has also been described using the Heckel equation (*Equation 5*), which predicts a linear dependence of the logarithm of tablet porosity versus compression pressure. In *Equation 5*, *K* and *B* are empirical constants.

$$-\ln(\textit{porosity}) = K \times (\textit{compression pressure}) + B \quad (5)$$

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However, the Heckel equation is overly simplistic in that it does not adequately describe powder compressibility in the low-pressure region, where ln(*porosity*) versus pressure data are not linear. More sophisticated models, such as the modified Heckel equation (*21*) and the Drucker-Prager Cap Model (*22*), have proven to be more reliable in describing powder compressibility to account for the transition between the state of a powder and the state of a tablet.

11. COMPACTIBILITY PROFILE

The relationship between tensile strength and solid fraction (or porosity) is termed "compactibility". Generally, tablet tensile strength increases exponentially with increasing solid fraction, and powder compactibility is often well described by the Ryshkewitch-Duckworth equation (*Equation 6*) where *k* and *A* are empirical constants (*23*):

$$\log(\textit{tensile strength}) = k \times (\textit{solid fraction}) + A \quad (6)$$

This relationship is qualitatively reasonable, as the presence of more or larger pores in a compact weakens it. Moreover, this relationship highlights the importance of determining tablet porosity to gain a better understanding of powder tableting performance. For example, when a low tablet tensile strength is associated with high porosity (solid fraction), an effective strategy for overcoming the tableting problem is to add a more highly deformable excipient, which increases the plasticity of the powder and decreases tablet porosity. See *Figure 6* for an example of a compactibility profile.

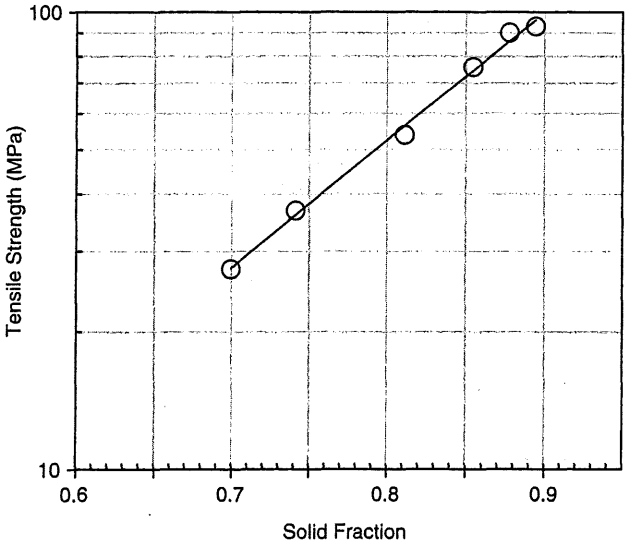

Figure 6. Example of a compactibility profile.

12. TABLET COMPRESSION PROFILE

Figure 7 illustrates the relationships among tensile strength, compression pressure, and solid fraction (or porosity) and related tableting parameters. The relationships among tensile strength, compression pressure, and solid fraction (or porosity) can be presented in three dimensions as shown in *Figure 8*, where the three faces of the three-dimensional plot represent the tabletability, compressibility, and compactibility (*24*).

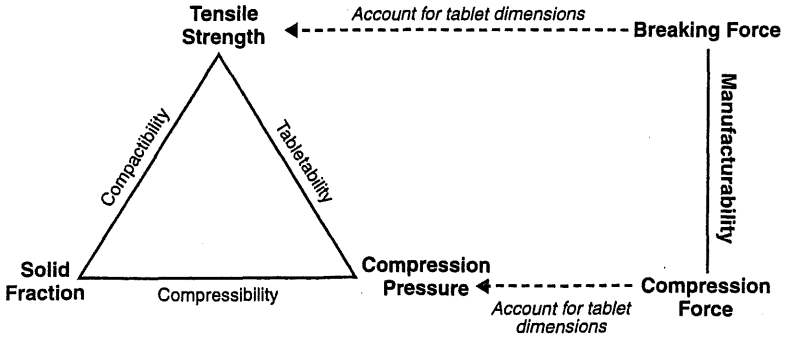

Figure 7. Relationships among tableting parameters for compression data analysis.

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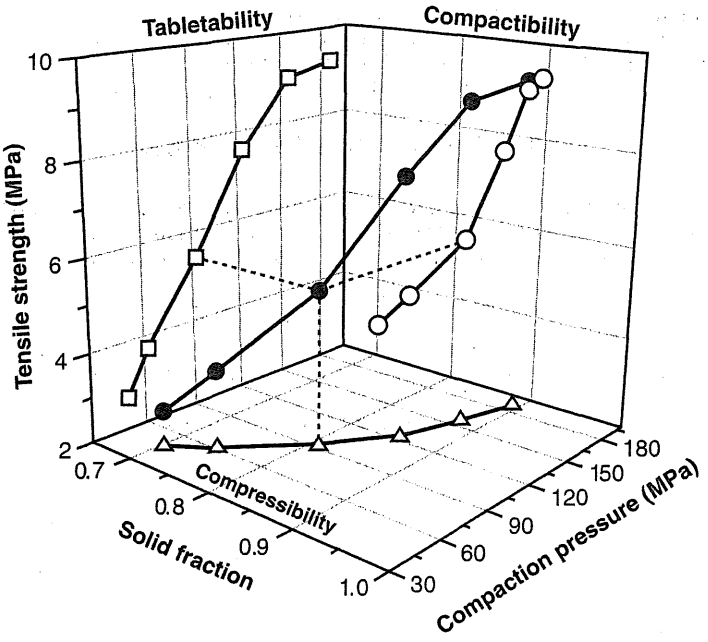

Figure 8. Three-dimensional compression profile.

13. MACHINE SPEED SENSITIVITY

The effect of compression speed may be assessed by comparing the properties of compacts that were prepared at different compression speeds. A range of compression speeds can be obtained by using a hydraulic press, a rotary tablet press, or a compaction simulator. For fundamental material assessments, tablet properties such as tensile strength, hardness, and porosity can be used to quantify strain rate sensitivity (*SRS*) of a powder using *Equation 7*.

$$SRS = \frac{\left| property_{(SR2)} - property_{(SR1)} \right|}{property_{(SR1)}} \qquad (7)$$

SR1 = low strain rates (e.g., low compression speed)
SR2 = high strain rates (e.g., high compression speed)

14. CONCLUSIONS AND RECOMMENDATIONS

Tablet compression is a complex process dependent on the drug product composition, material properties, manufacturing process parameters, environment conditions, equipment, and tooling design. To gain an in-depth understanding of the compression behavior of a powder, it is beneficial to have knowledge of the particle size and shape, solid form, and water content of the powder. Without appropriate control and descriptions of these factors, the data obtained are not meaningful for accurately characterizing tablet compression properties of a powder. Therefore, these properties should always be considered during the analysis of the tablet compression results. *Table 1* shows examples of parameters that are commonly used in the characterization of the compression properties of pharmaceutical solids.

Table 1. Important Parameters to Specify When Characterizing Powder Compression Properties

Experimental Parameter	Parameter Value(s) Used	Example Parameters in Common Use[a]
Tooling type	Specify	Round flat-faced; standard round concave
Tooling size	Specify	8 mm, 10 mm, 13 mm
Compression speed	Specify	Tablets/minute, 0.03 mm/s, 300 mm/s
Punch displacement–time profile	Specify	Saw tooth, square, sinusoidal; single-sided, double-sided
Compression pressure range	Specify	25–300 MPa
Solid fraction range	Specify	0.6–0.95
Tablet properties (weight, dimensions)	Specify	Tablet thickness, tablet diameter
Powder equilibration	Specify	20°, 40% relative humidity (RH)

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Table 1. Important Parameters to Specify When Characterizing Powder Compression Properties *(continued)*

Experimental Parameter	Parameter Value(s) Used	Example Parameters in Common Use[a]
Lubrication	Specify	Type; external die; internal, %
Tablet storage (time, temperature, and RH)	Specify	None; 24 h at 20°, 40% RH
Tablet press configuration	Specify	With or without pre-compression setting
Data Analysis		
Compressibility	Specify [NOTE—If possible, the entire curve is preferred.]	Compression pressure (σ_c) at specified solid fraction (SF)
Compactibility		Tensile strength (σ_x) at specified SF
Tabletability		σ_x at specified σ_c, or σ_c at specified σ_x
Ejection force		Ejection force at specified σ_c
SRS	Specify	*SRS* = (P2 – P1)/P2, P2 = mean yield pressure at 300 mm/s; P1 = mean yield pressure at 0.033 mm/s, saw-tooth punch displacement–time profile

[a] These examples are not required and they may not be suitable for all materials. They should not be viewed as prescribed values for characterizing powders.

GLOSSARY

Breaking force: The force [in Newtons (N)] required to cause tablet mechanical failure. Often referred to as tablet hardness (see ⟨1217⟩).

Brittleness: The property that leads to particle or compact fracture, typically very rapidly.

Capping: Laminar splitting along the edge of the crown or band of a compressed tablet.

Compactibility: The ability of a powder to form an intact compact with measurable strength.

Compactibility profile: Change in tensile strength of a compressed body with solid fraction (or porosity).

Compaction: The transformation of a powder into an intact compact with measurable strength and defined shape by the application of compression pressure. Usually used synonymously with consolidation.

Compaction emulator: A device that physically approximates tablet press configurations where compression parameters may be applied; parameters may include pre-compression and compression roll dimensions, tableting speed, ejection angle, and punch design.

Compaction simulator: A device that permits powder compression, typically using a hydraulic source to control displacement of punches that may be designed to match the force displacement profile of a high-speed press by means of a computer.

Compressibility: The ability of a powder to be compressed (reduced in volume) by the application of stress.

Compressibility profile: Change in solid fraction (or porosity) of a compressed body with applied pressure.

Compression: The reduction in volume of a powder bed due to the application of a stress, e.g., loading.

Compression force: Force applied to compress a powder bed. The unit kN is commonly used in tableting.

Compression pressure: Pressure (force/area, in MPa) applied to specimen material; sometimes used interchangeably with the terms "compaction pressure" or "compression stress". See *Compaction* and *Compression*.

Compression profile: The relationship between *Compression pressure*, *Solid fraction* (or porosity), and tensile strength.

Dwell time: Duration of time (in ms) that the compression roll is in contact with the flat portion of the punch head. Often used to describe rotary compression processes with a modified sinusoidal punch displacement–time profile.

Elastic deformation: The change in shape of a stressed body that is completely recovered when stress is released. This is time-independent, recoverable deformation.

Elastic limit: The amount of stress at which a material deviates from linear elastic behavior, i.e., the smallest stress that leaves a detectable permanent deformation when unloaded.

Failure: The permanent collapse, breaking, or deforming of the material.

Force: A push or pull (in N) resulting from the interactions between two objects.

Hardness: See *Breaking force*.

Hydraulic press: A device that uses liquid pressure to enable the application of force to a specimen.

Indention hardness: The resistance of a surface to permanent deformation (indentation) when subjected to pressure by a hard object.

Lamination: Condition in which a tablet splits or separates into layers.

Manufacturability profile: Change in breaking strength of a compressed body with applied force.

Mechanical properties: The characteristics of a material upon application of a stress. Examples include tensile strength, yield strength, plasticity, brittleness, hardness, elastic modulus, and bendability.

Plastic deformation: The permanent change in shape of a solid body, without fracture, resulting from the application of sustained stress beyond the elastic limit. Deformation occurs without a change in particle volume.

Plasticity: See *Plastic deformation*.

Pressure: Force applied to a unit area (in MPa), used interchangeably in this chapter with stress.

Porosity or void fraction: A measure of the empty spaces in a material. This is the fraction of the voids divided by the total volume. Porosity ranges between 0 and 1, or as a percentage between 0% and 100%.

Shear stress: The force per unit area (in MPa) acting along a plane through a body.

Solid fraction: The apparent density divided by the absolute density of the solid, sometimes referred to as relative density. Solid fraction = (1 – porosity).

Sticking: The adherence of material to the faces of tablet press punches or dies after compression.

Stress: Normal stress. Used interchangeably in this chapter with pressure. See *Pressure*.

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Tabletability profile: Change in tensile strength of a compressed body with applied pressure.
Tablet press: A mechanical device that compresses powder into tablets of desired size and weight.
True density: The average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement.
Viscoelastic deformation: Time-dependent partially recoverable deformation.
Yield strength: The stress needed to produce a specified amount of plastic deformation (usually a 0.2% change in length).

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⟨1063⟩ SHEAR CELL METHODOLOGY FOR POWDER FLOW TESTING

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1. INTRODUCTION

A large number of pharmaceutical processes involve powder transfer and feeding, and the ability of powders to flow in a controlled manner during these operations is critical to product quality. For example, drug product attributes such as weight and content uniformity depend on powder flow characteristics. Shear cells are among the most important methods for measuring powder flow properties, and the data from shear cell testing can be used to predict a wide variety of powder flow behaviors during pharmaceutical manufacturing. Shear cells have many advantages over simpler methods of measuring powder flow (see *Powder Flow* ⟨1174⟩), but their operation is more complex and the procedures for their use must be carefully controlled to produce accurate and reproducible data. This chapter describes best practices for obtaining reliable and accurate powder flow data using a shear cell.

1.1 Scope

This chapter focuses on the three most popular shear cell types used for measuring powder flow properties:

1. Translational (Jenike type)
2. Annular (Schulze type)
3. Rotational (Peschl type)

These three shear cell types are categorized as direct shear tests in which a region of the powder is sheared under a series of controlled normal stresses. From these data, a wide variety of parameters can be obtained, including the yield locus representing the shear-stress to normal-stress relationship at incipient flow, the angle of internal friction, the unconfined yield strength, powder cohesion, and a variety of related parameters such as the flow function. In addition, these three shear cells can be set up with wall coupons (see *Appendix*) to measure the powder wall friction. When the shear cell data are combined, they can be used for bin and hopper evaluation and design. Other testing approaches, such as triaxial testers and indirect or hybrid testers (e.g., the Johanson indicizer), are outside the scope of this chapter.

2. THEORY AND PRINCIPLES

The flow behavior of a powder is fundamentally different from the flow of a fluid. First, powder flow properties and shear behaviors are strongly dependent on the consolidation stresses applied to the powder and are minimally dependent on the strain or flow rate (under the assumption of quasi-static conditions such as flow in a bin). Fluid flow, in contrast, is strongly dependent on the strain rate (where viscosity describes the relationship between shear stresses and strain rates) and is minimally dependent on absolute pressure. Second, when shear stresses are applied to powders, they may not immediately fail (i.e., they can avoid flow under a sustained shear stress), whereas Newtonian and viscoelastic fluids do not behave this way and always flow under an applied shear stress. Thus, powders have the potential for arching and rat-holing, depending on the flow pattern (see *Appendix*).

However, powders can sustain a shear stress without flowing only up to a certain point. The yield locus for a given powder is a function of many variables, including its composition, particle size and shape, moisture content, temperature, time stored at rest, and the state of consolidation. Once a powder is subjected to stresses (whether by gravity or some mechanical means) that reach or exceed the yield locus, the powder flows. Hence, determining the yield locus for a given powder under conditions representative of its manufacturing process is an essential step in evaluating the flow behaviors for that process. In some circumstances, this may involve testing under controlled environmental conditions, as well as holding the powder under load for an extended period before shearing (a "time test").

Because powder properties are highly dependent upon the degree of consolidation, the preparation of a uniform powder bed (consistent bulk density throughout the powder bed) is the first critical step of shear cell testing. The next stage of testing is the application of a normal stress (σ) and shear stress (τ) to the powder bed to achieve steady-state shear, resulting in a known state of consolidation. The shear stress then is removed, and a reduced normal stress is applied. A shear stress then is applied and is progressively increased until the powder bed yields and begins to flow. This procedure is repeated at several different normal stress conditions to create a "yield locus" plot. To complete a full flow function analysis, the operator must determine several yield loci, which requires that the unconfined yield strength be determined under several different levels of consolidation.

Although this chapter focuses on the powder (particle–particle) properties, the wall friction (particle–wall) properties and bulk density are also important. Such properties are used for bin design and also are essential when one compares different wall materials (e.g., different grades and finishes of stainless steel, or the effect of plastic coatings on powder flow behaviors). The most fundamental property of a wall material in this regard is Φ', the angle of friction between the bulk powder and the wall material, or, correspondingly, the coefficient of wall friction (μ_w):

$$\mu_w = \tan(\Phi') = \tau_w/\sigma_w$$

τ_w = wall shear stress

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σ_w = wall normal stress

Note that Φ', and hence μ_w, often are a function of the applied normal stress (σ_w).

3. DESCRIPTION OF SHEAR CELL COMPONENTS AND DESIGNS

Figures 1, *2*, and *3* provide schematics of the different types of shear cells considered in this general chapter. All have the same general operating principle of being able to measure the force required to shear a powder bed to which a normal load has been applied. The applied load or measured force can be expressed as a stress by dividing by the cross-sectional area of the shear plane under consideration.

The translational shear cell (*Figure 1*) has a fixed base with a movable ring above it, and both hold the bulk powder. A cover that fits within the ring is used to contain the powder and to provide a uniform application of the normal load (*N*). The ring and cover are pushed as a unit while a load cell records the shear forces (*F*) that are generated. The shear plane forms between the powder that is contained in the base and the powder contained in the ring.

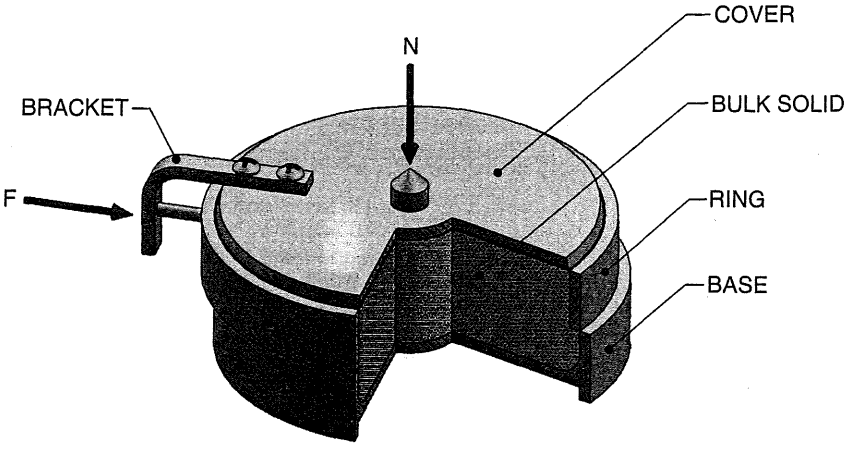

Figure 1. Descriptive schematic of the translational shear cell.

The annular shear cell (*Figure 2*) consists of a shear cell or base that holds the powder. A cover that fits within the cell is used to contain the powder and to provide a uniform application of the normal load (*N*). The cover is free to move up and down, but otherwise remains fixed in place by a load cell that measures the shear forces (*F*) that are generated. The shear cell is rotated at a constant angular velocity (ω) to create a shear plane that forms in the powder bed somewhere between the cell bottom and the cover. The cover and shear cell usually have baffles or other surface features that prevent the powder from sliding or shearing at the interface between the powder and the cover or shear cell base.

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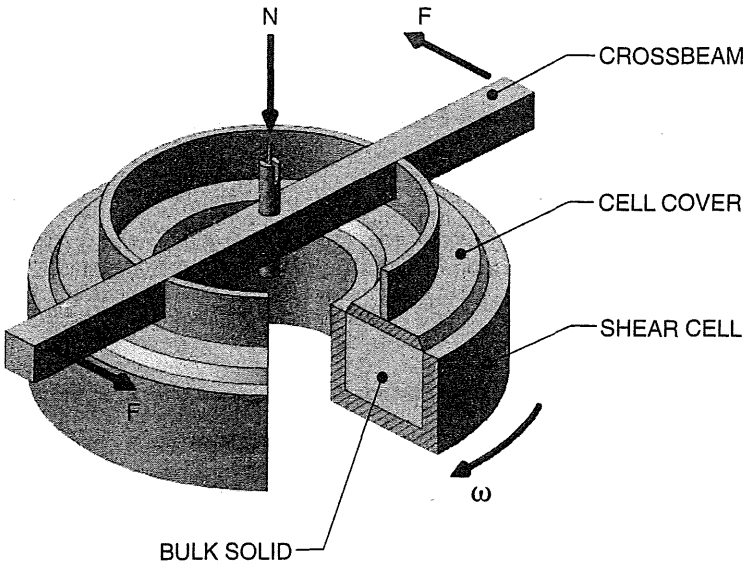

Figure 2. Descriptive schematic of the annular shear cell.

The rotational shear cell (*Figure 3*) has a base and ring that hold the powder. A loading lid that fits within the ring is used to contain the material and to provide a uniform application of the normal load (*N*). The loading lid is free to move up and down, but otherwise remains fixed in place, connected to a load cell that measures the shear forces (*F*) that are generated. The shear cell base then is rotated at a constant angular velocity (ω) to create a shear plane that forms in the powder bed somewhere between the ring and the base. Alternatively, the base can be fixed, and the lid can be rotated to create the shear plane in the powder specimen. The ring and base usually have surface features that prevent the powder from sliding at the powder-surface interface.

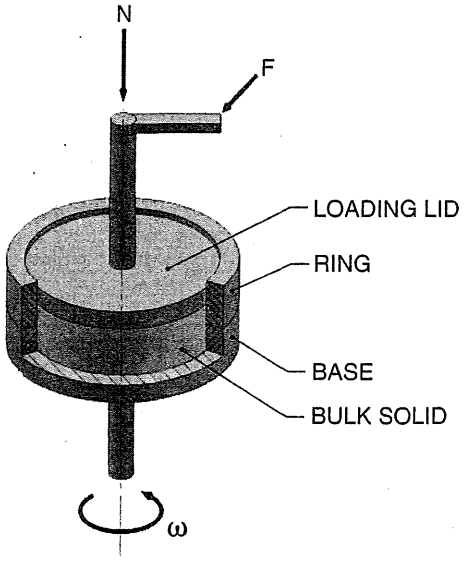

Figure 3. Descriptive schematic of the rotational shear cell.

The choice of one shear cell type over another depends on factors such as the availability of equipment and ease of use for a particular technician. Because of the infinite travel permitted with the annular and rotational shear cell geometry, these types of cells are more suited for powders for which reaching steady state requires large strains. Another advantage of the infinite travel of rotational testers is that a preconsolidation step may not always be required.

Wall friction tests can be carried out using all of the shear cells referenced in this chapter with only minor modifications, namely by creating a shear plane in which the powder slides over a suitable wall coupon rather than having the shear plane within the powder bed. This usually is achieved by replacing the base of the shear cell with a coupon of the wall material and adjusting the amount of powder used to create a shear plane at the powder–coupon interface.

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4. SHEAR CELL MEASUREMENTS

4.1 Sample Preparation

The applicability of shear cell data depends strongly on the use of a representative powder sample. Detailed discussion of appropriate sampling procedures can be found in *Bulk Powder Sampling Procedures* ⟨1097⟩.

The cell size (volume and diameter) usually is selected based on the maximum particle size of the powder sample, and the proportion of large particles in the sample. The particle size limit is usually reported by the supplier of commercially available testing equipment. For a translational cell, the maximum particle size is approximately 5% of the diameter of the cell, and for an annular cell the maximum particle size is approximately 10% of the width of the annulus (i.e., the difference between the outer and inner radius of the shear cell trough). Fresh powder is desired for each test, if possible, so the use of a smaller cell may be preferable to reusing the powder. Under some circumstances, a smaller cell may be needed to reach higher consolidation stresses. Smaller cell sizes may have reduced precision and increased bias, depending on the specific tester and the cell size under consideration, because of the larger contribution of non-idealities caused by wall effects.

The shear strength of some powders (e.g., fibrous or flaky solids) is often caused by interlocking of particles; therefore, such materials may prove to be unsuitable for testing with a conventional shear cell. Likewise, powders with high springback (rubber-like or highly elastic properties) sometimes can fall outside of the practical limits of a shear tester. Poor reproducibility and unexpected results may indicate a problem with testing these types of powders.

Powder samples must be handled and tested under conditions that are relevant from a practical standpoint. For example, many pharmaceutical powders will sorb/desorb moisture from their surroundings according to the ambient relative humidity and temperature. This sorbed moisture can significantly affect the measured powder flow properties. Controlling the environmental conditions during sample handling and testing so that sample behavior is representative of the processing conditions of interest is essential for most powders.

Some pharmaceutical powders "age" quickly after they are produced, and such powders may need to be tested immediately after they are manufactured. The testing of some powders may result in caking or particle attrition that can render a powder unsuitable for retesting. In these circumstances, fresh powder samples should be used for each test.

4.2 Instrument Preparation

The shear cell should be situated in an area that is free of vibrations. Vibrations can affect the instrument readings and also can densify or dilate the powder while it is being tested.

Because shear testers measure forces, their load cells must be calibrated for the forces being measured. Some testers also measure the displacement (linear or angular) of the cell (indicating travel) and/or the vertical displacement of the cover or lid (used to calculate the volume of the cell and hence an average bulk density). In these cases, the displacement transducers must also be calibrated. The rate of any changes in force and displacement will need to be confirmed as part of this calibration. In addition, the shear cell is operated under the assumption that all of the parts are precisely aligned and there is a minimal degree of wobble when the cell rotates. For example, if the cover and shear cell trough are not parallel during testing, it will be difficult to get reproducible results. Thus, the alignment and mechanical operation of the shear cell must be evaluated at regular intervals. No other adjustments with respect to calibration are performed, although the correct performance of the apparatus may be confirmed by the measurement of the yield locus of a reference powder under standardized test conditions.

Wall coupons used for testing must be representative of the surfaces upon which the powder will slide. Directional surfaces can be oriented in the direction of flow in the application (e.g., grain oriented down the length of a hopper), oriented in the worst-case condition (often, grain oriented perpendicular to the direction of flow in the hopper), or evaluated in both directions.

All shear cell components (including wall coupons) should be carefully cleaned before use with a method that does not abrade the surface or leave any chemical residue behind. In addition, the cleaning method should be capable of removing all of the components of the test sample, including lubricants and other additives.

5. SELECTION OF TEST CONDITIONS

Preshear normal stresses usually are selected based on the powder's density values. Standards, such as ASTM Standard 6128, provide tables of specific initial preshear normal stresses as a function of the sample bulk density.[1] Subsequent preshear normal stresses are given in multiples of the initial preshear value (e.g., 2, 4, and 8 times the initial level). The number of preshear normal stress levels should be at least 4. It is often valuable to match preshear stresses in the test to the stresses expected in the processing situation of interest. Hold times for time tests are selected based on matching the hold time in the practical application. If long hold times are unrealistic, operators may consider several intermediate time points and extrapolation of the data.

Normal stress levels for shear are selected to provide a range of data points on the yield locus. Typically, a range between 25% and 80% of the preshear normal stress is valid, although powders with high unconfined yield strength or internal friction may require a narrower range. The range of normal shear stress levels should be sufficient to allow meaningful fitting and extrapolation of the data to determine the unconfined yield stress and other related parameters.

[1] ASTM. D6128-06. Standard test method for shear testing of bulk solids using the Jenike shear cell. West Conshohocken, PA: ASTM; 2006.

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6. TEST PROCEDURE

Shear cell testing involves a sequence of steps that consolidates the powder to a known extent and then shears it under carefully controlled conditions while recording the applied normal stresses and measuring the shear stresses. In most cases, the essential steps are as follows:

1. Fill the test cell with an appropriate and representative sample of the powder in a manner that provides a uniform bulk density and composition. This sample in the test cell is referred to as the "specimen". Detailed procedures vary according to the type of tester being used, but in all cases it is essential to evenly distribute the powder and avoid pockets of air that can be difficult to remove later.
2. For translational shear cells, perform a preconsolidation step to create the desired density in the cell. This is accomplished by twisting the cover while under a compressive normal load. This step reduces the travel needed to achieve a steady-state preshear value and can limit the total vertical displacement of the cover during the test. A preconsolidation step may be conducted in other cell types, but this is often not conducted, because the unlimited rotation of the annular and rotational cells can generally provide a sufficient state of consolidation. The appropriate level of preconsolidation is critical, and care must be taken not to over- or under-consolidate the specimen.
3. Consolidate the specimen by applying a known normal stress to the powder specimen via the cell cover/lid.
4. Preshear the specimen until a steady-state shear value is reached. Care should be taken to avoid over-consolidating the specimen. The applied shear stress is then reduced to zero.
5. An instantaneous shear test is run by shearing the specimen under a reduced normal stress (with respect to the applied preshear stress) until the shear stress goes through a maximum value and then begins to decrease.

Steps 1–5 are repeated at a series of different reduced normal stress conditions to create a complete set of data (yield locus) and then at a series of different preshear normal stress conditions to create a "flow function". The test cell is preferably emptied and refilled before generating each point on the yield locus. For annular or rotational shear cell types, it is common that steps 3–5 are repeated a number of times for the same specimen without emptying and refilling the cell. If the same specimen is used for multiple test points, caution must be taken to ensure that the specimen has not changed from test to test.

A wall friction test is run in an analogous manner by sliding the specimen over a coupon of wall material and measuring the shear stress as a function of the applied normal stress. In a time test, a normal stress is applied to the specimen for a predetermined period of time before shearing. Both wall-friction and time-consolidation tests can be conducted with the three types of shear cells described in this chapter, as long as directionality (if any) of the wall surface, relative to the cell movement, is taken into account.

7. DATA ANALYSIS AND CALCULATIONS

Illustrative results from the shear cell test procedure are presented in *Figure 4*. For analysis, the applied preshear normal stress (σ_p) and all valid applied normal stress points (σ_{si}) for $I = 1, 2, 3, \ldots, n$ and their corresponding measured shear stress points[2] ($\tau_p, \tau_{s1}, \tau_{s2}, \ldots, \tau_{sn}$) are plotted in ($\sigma, \tau$)-coordinates. These data are then used to generate a smooth line through all the valid shear points to obtain the yield locus. Typically, the yield locus passes above or through the preshear point. If not, the test results should be analyzed in more detail. In some circumstances, the yield locus can be forced to pass through the preshear point and can be fitted to all yield points.

The "unconfined yield strength" of the powder is obtained by drawing a Mohr circle through the origin and tangential to the yield locus. The higher point of intersection of this Mohr circle and the σ-axis is the unconfined yield strength f_c. The "major consolidation stress", σ_1, is found by drawing a second Mohr circle through the preshear point and tangential to the yield locus. The higher point of intersection of this Mohr circle (consolidation locus) and the σ-axis is the major consolidation stress.

[2] For a given normal stress applied, the measured steady-state shear stress values should be consistent from test to test. To account for normal experimental variations, a given measured shear stress value may be prorated in an appropriate fashion, relative to the steady-state value associated with that point.

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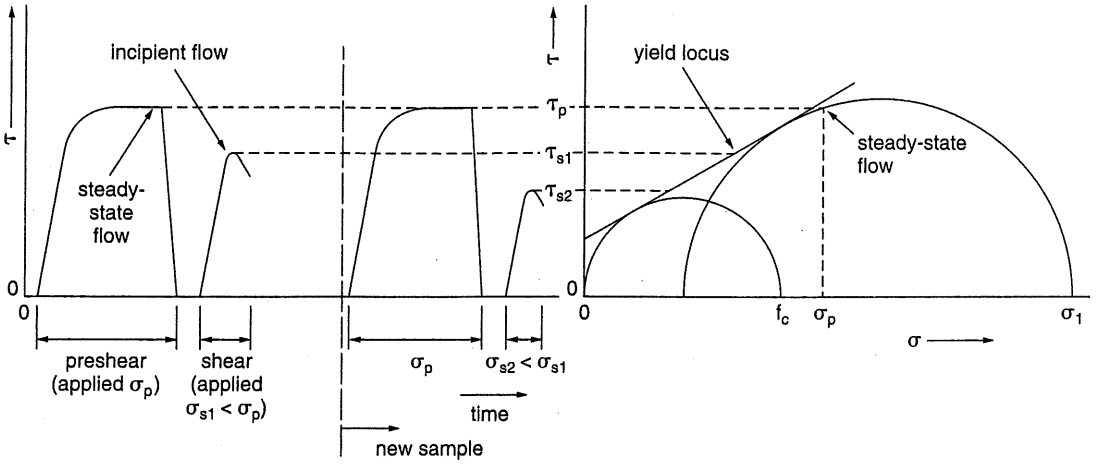

Figure 4. Shear cell testing and data analysis: shear cell raw data (*left*) and yield locus calculated from raw data (*right*).

The "angle of internal friction", Φ, is defined as the slope of the yield locus, as shown in *Figure 5*. A line drawn through the origin and tangential to the steady-state Mohr circle has an angle, δ, that is defined as the "effective angle of friction". The "cohesion," C, is the intersection of the yield locus and the τ-axis.

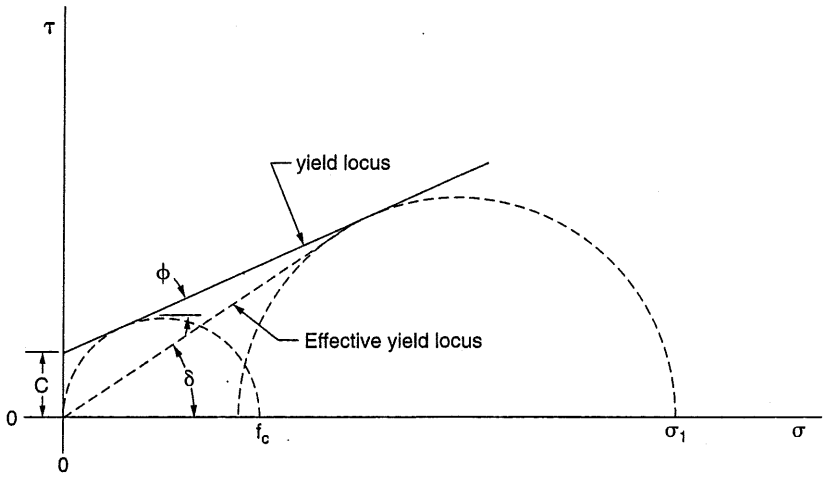

Figure 5. Graphical representation of the angle of internal friction (Φ), effective angle of friction (δ), and cohesion (C).

From a family of yield loci generated at different preshear normal stresses, it is possible to plot the unconfined yield strength, f_c, as a function of the major consolidation stress, σ_1, as shown in *Figure 6*. The best-fit curve to these points is called the "flow function" and can be used to calculate the potential for arching and rat-holing in a storage bin.

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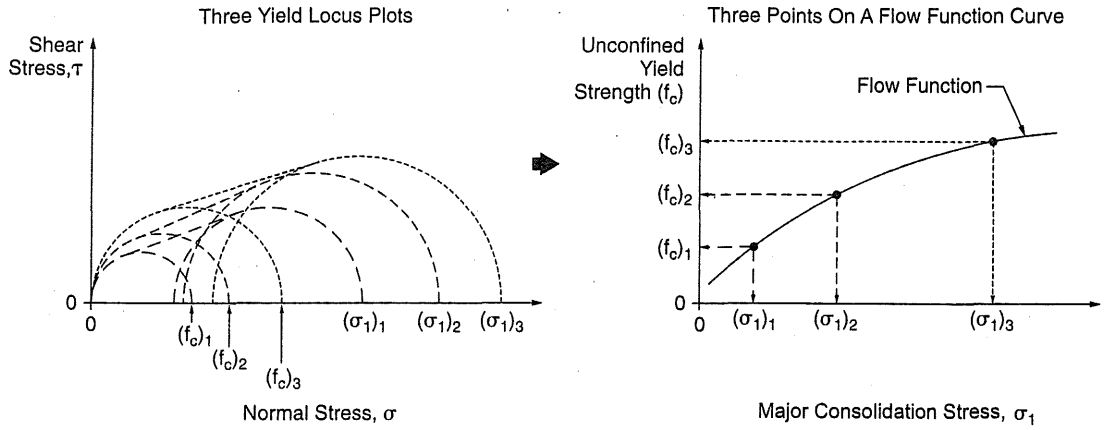

Figure 6. Calculation of the flow function from multiple yield locus plots.

The "kinematic wall yield locus" is developed in a manner similar to the calculation of the yield locus. This is depicted in *Figure 7*. The "wall friction angle", Φ', is defined as the inclination of a line from the origin to a point on the wall yield locus. In general, low wall friction angles are desirable.

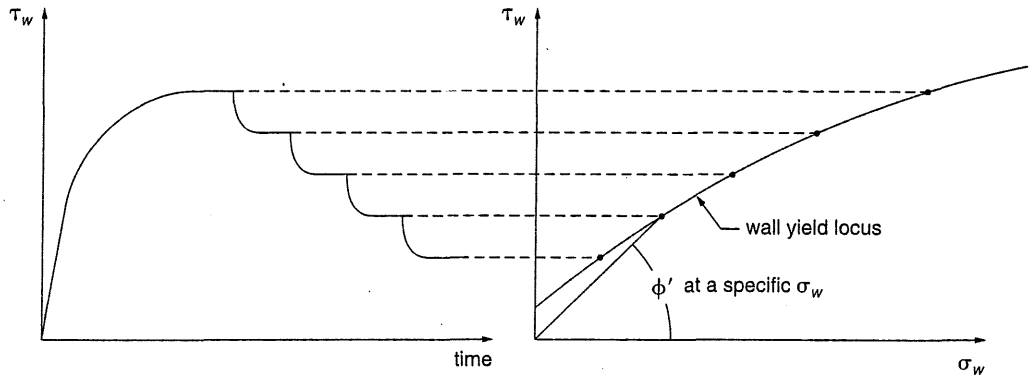

Figure 7. Calculation of the wall yield locus.

Test conditions should be carefully documented for every shear cell analysis to allow for accurate data interpretation and meaningful data comparisons. When analysts compare multiple powders, matching the tester type, specific tester model, test cell size, sample preparation, test procedures, applied normal stresses, and environmental conditions provides increased confidence that any differences in the results are attributable to the powder and not to the test. Changes in material sourcing or manufacturing may require the repetition of the initial studies.

Shear cell tests allow the analyst to characterize consolidated powders under quasi-static conditions. The tests are unable to directly measure powder flow properties at very low stress conditions or high shear rates, which may occur in some powder-handling situations. Thus, no single parameter can describe powder flowability. Instead, the parameters described in this chapter should be interpreted together and in the context of the conditions under which the powder will be stored and handled (e.g., equipment dimensions, environmental conditions, and others).

The ratio of the major consolidation stress, σ_1, to the unconfined yield strength, f_c, at a particular value of the major consolidation stress provides a general, simplified way of assessing powder flowability. As a general case, the flow of powdered materials is classified in *Table 1*.

Table 1. General Classification of Flow Character[a]

σ_1/f_c	Flow Character
<2	Very cohesive, nonflowing
2–4	Cohesive
4–10	Easy-flowing
>10	Free-flowing

[a] Jenike A. Storage and flow of solids: bulletin no. 123 of the Utah Engineering Experiment Station. Salt Lake City, UT: University of Utah; 1964.

This approach can be useful for rank ordering the flow character of pharmaceutical powders as part of formulation and process development activities. However, it should be noted that this simplified view may lead to significant errors in

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interpretation because this approach does not take into account the flow pattern or the size or geometry of the manufacturing equipment being used.

8. REFERENCE MATERIALS AND REPRODUCIBILITY

For a tester that is installed and operated correctly, the performance of the instrument and operator can be assessed by testing a standard material. There is currently no specific pharmaceutical reference material for shear cell test method verification. Limestone powder has been used as a certified reference material.[3]

Figures 8, *9*, *10*, *11*, *12*, and *13* show representative yield loci and flow function plots for several common pharmaceutical excipient powders determined at a range of conditions. The plots are provided to illustrate the type of data that can be generated for pharmaceutical powders using a shear cell and the typical reproducibility of the data. The data in *Figures 8*, *9*, *10*, and *11* were collected using a procedure consistent with that described for *Figure 4*. These plots are not intended for use as reference data, and they should not be assumed to be representative of all test conditions and material types.

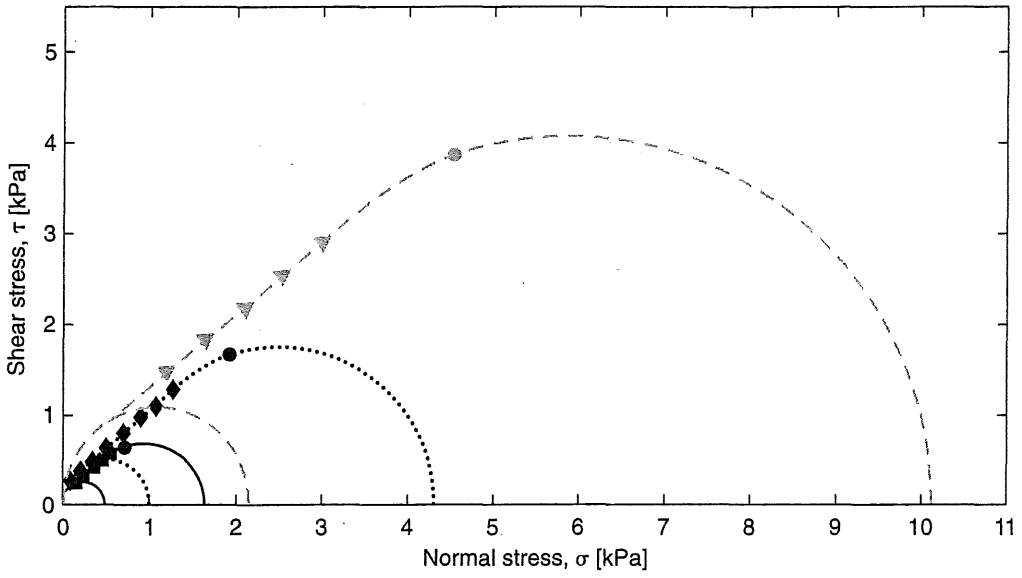

Figure 8. Representative yield loci for microcrystalline cellulose NF (Avicel PH-101) at preshear stresses of 730 Pa (*solid*), 1.9 kPa (*dot*), and 4.6 kPa (*dash*).

[3] Limestone powder certified reference material (BCR-116) is available from the Institute for Reference Materials and Measurements of the European Commission and instrument suppliers.

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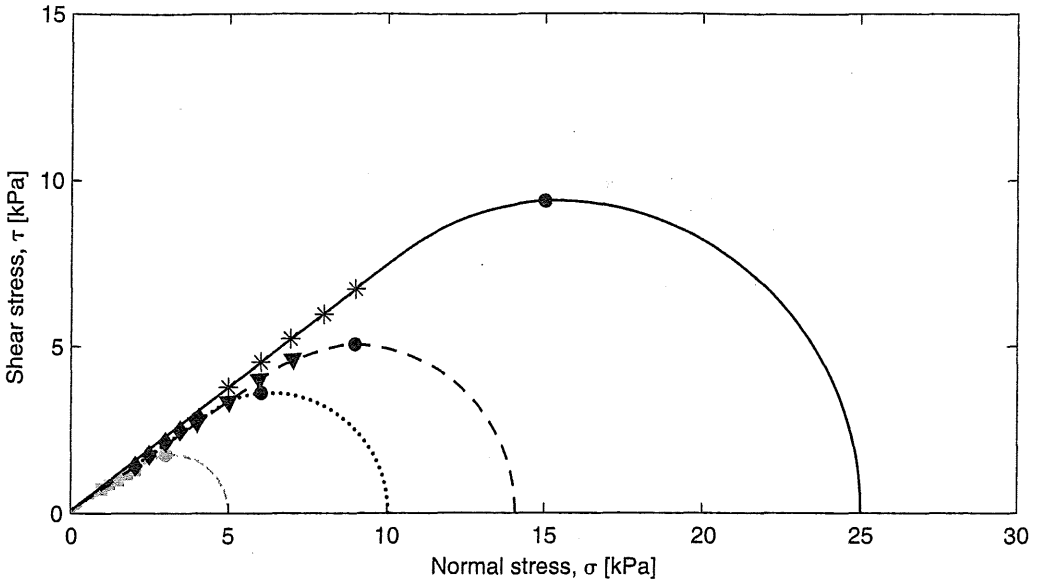

Figure 9. Representative yield loci for sorbitol at preshear stresses of 3 kPa (*gray, dash*), 6 kPa (*dot*), 9 kPa (*black, dash*), and 15 kPa (*solid*).

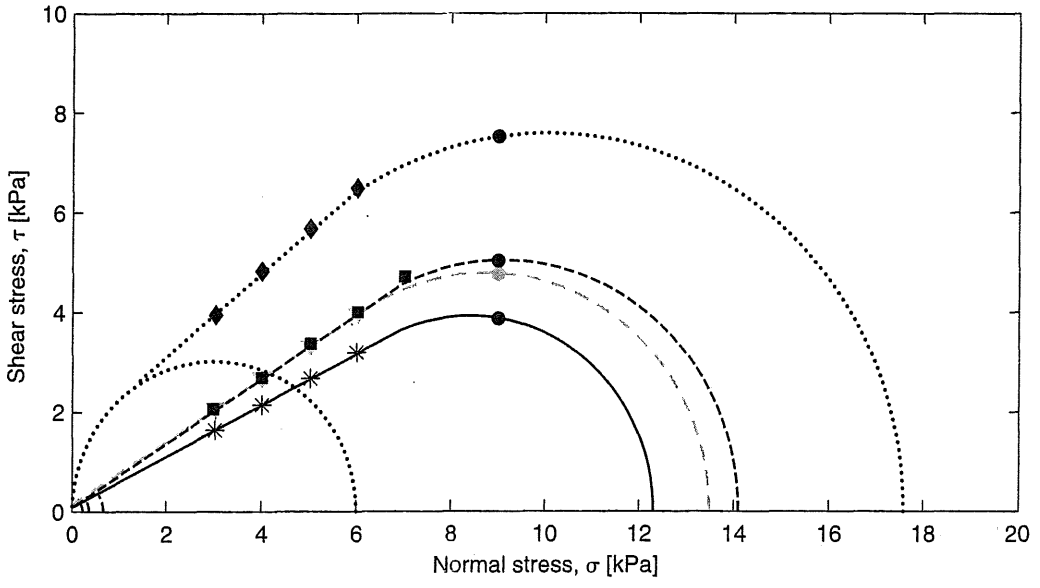

Figure 10. Representative yield loci for mannitol (*dot*), sorbitol (*black, dash*), sieved lactose (*gray, dash*), and spray-dried lactose (*solid*) at a preshear stress of 9 kPa.

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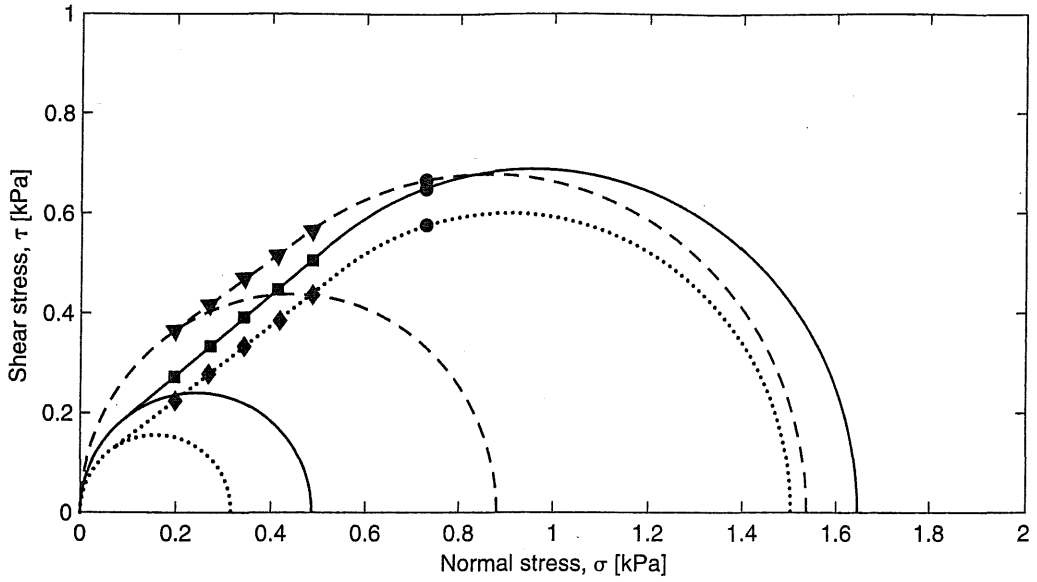

Figure 11. Representative yield loci for several different grades of microcrystalline cellulose NF. Avicel PH-101 (*solid*), Avicel PH-102 (*dot*), and Avicel PH-105 (*dash*) at a preshear stress of 730 Pa.

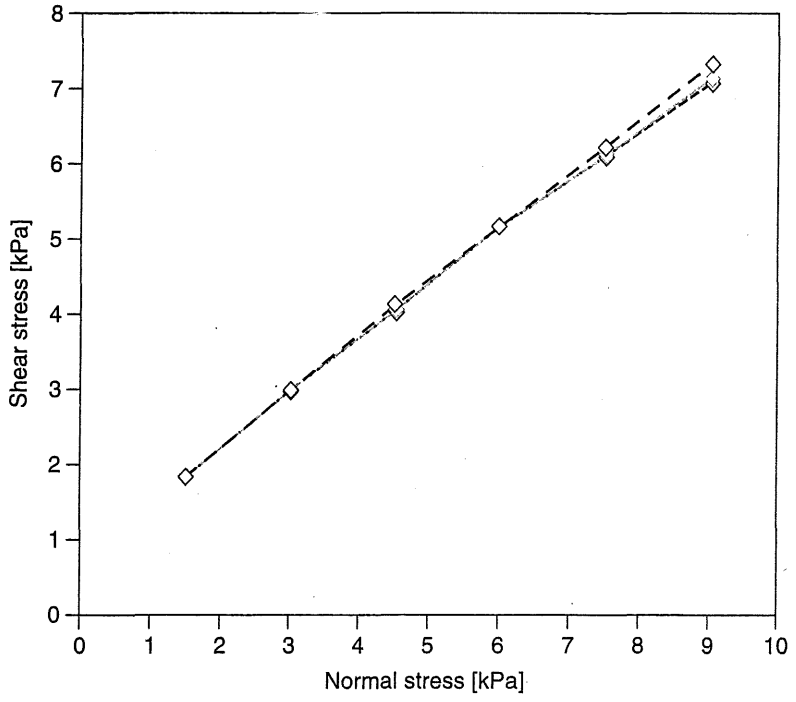

Figure 12. Representative yield loci for microcrystalline cellulose (Avicel PH102) from three independent determinations conducted at a preshear stress of 9 kPa. [Figure adapted from Sun CC. Setting the bar for powder flow properties in successful high speed tableting. *Powder Technol.* 2010;201(1):106–108.]

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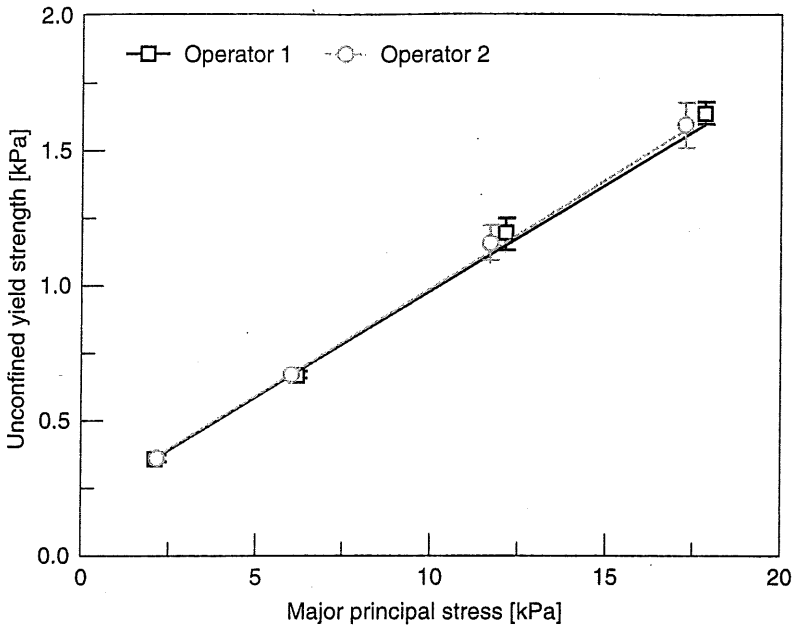

Figure 13. Representative flow function plots for microcrystalline cellulose (Avicel PH102) from two independent operators. [Figure adapted from Shi L, Chattoraj S, Sun CC. Reproducibility of flow properties of microcrystalline cellulose—Avicel PH102. *Powder Technol.* 2011;212(1):253–257.]

APPENDIX

Notation

Term	Symbol and SI Units	Definition	Comments
Angle of internal friction	Φ, degrees	The inclination of the yield locus (*Figure 5*).	
Angular velocity	ω, degrees per second	The angular velocity at which the shear cell base rotates.	Is usually held constant.
Annular shear cell	N/A	A shear cell based on a rotating annulus design (*Figure 2*).	The Schulze Ring Shear Tester is a common annular shear cell.
Arching	N/A	The formation of a bridge of powder across an opening caused by, for example, attractive interactions between particles.	Also known as "bridging".
Bulk density	ρ, kg/m^3	The mass of a quantity of a powder divided by its total volume.	Varies with the applied normal stress and history of a sample.
Cohesion	C, *Pa*	The failure shear stress at zero normal stress, normally obtained by extrapolation of the yield locus (*Figure 5*).	An indication of the intrinsic strength of an unconfined powder.
Consolidation	N/A	The process of increasing the density of a powder, which usually results in increasing its unconfined yield strength. Achieved in a shear cell by applying a preshear normal load and shear load to the specimen.	
Coupon	N/A	Flat surface that is in contact with powder specimen during wall friction testing.	The coupon surface finish must be representative of the wall surface of interest.
Effective angle of friction	δ, degrees	The inclination of the effective yield locus (*Figure 5*).	Sometimes used as a measure of relative flowability.
Effective yield locus	N/A	The straight line passing through the origin of the $\sigma - \tau$ plot and tangential to the steady-state Mohr's circle, corresponding to steady-state flow conditions of a powder of given bulk density (*Figure 5*).	
Flowability	N/A	A qualitative estimate of the relative flow properties of a powder.	Often based on measured values of the flow function or effective angle of friction.
Flow function	N/A	The plot of unconfined yield strength versus major consolidation stress for one specific powder (*Figure 6*). Quantified as the ratio of the major consolidation stress to the unconfined yield strength at a particular value of major consolidation stress.	

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Notation *(continued)*

Term	Symbol and SI Units	Definition	Comments
Funnel flow	N/A	A flow pattern where an active flow channel forms through stagnant material.	
Lid	N/A	Cover of the cell containing the specimen within a shear cell.	The lid position can be measured to determine displacement, bulk density, and powder dilation.
Major consolidation stress	σ_1, *Pa*	The major principal stress given by the steady-state Mohr circle. This circle is tangential to the yield locus and effective yield locus and passes through the preshear point (*Figure 4*).	
Mass flow	N/A	A flow pattern in a converging hopper where all material is in motion, including material sliding along the hopper walls.	
Mohr's circle	N/A	A graphical representation of a state of stress in coordinates of normal and shear stresses (*Figure 4*).	
Normal stress	σ, *Pa*	The stress acting normally (perpendicularly) to the considered plane.	σ_p is the preshear normal stress; σ_{si} for $i = 1, 2, 3, \ldots$ the test normal stresses.
Preconsolidation	N/A	Normal stress applied to consolidate a powder before testing in some shear cells (*Figure 4*).	
Preshear	N/A	Application of normal stress while shearing to attain steady-state shear conditions (*Figure 4*).	
Rat-holing	N/A	Tendency of powder to flow only in the region above an opening, thus forming a narrow rat hole in the powder.	Also known as "core flow".
Rotational shear cell	N/A	A shear cell operating under rotational shear conditions (i.e., rotational shearing perpendicular to the applied normal stress) (*Figure 3*).	The Peschl and Freeman shear cells are common rotational shear cells.
Shear stress	τ, *Pa*	The stress required to shear the powder in a direction perpendicular to the normal stress.	
Time consolidated strength	f_{ct}, *Pa*	The unconfined yield strength of a powder after being held at fixed consolidation conditions for a certain time.	
Time yield locus	N/A	The yield locus of a powder that has remained at rest under a given normal stress for a certain time.	
Translational shear cell	N/A	A shear cell operating under translational shear conditions (i.e., linear shearing perpendicular to the applied normal stress) (*Figure 1*).	The Jenike shear cell is a common translational shear cell.
Unconfined yield strength	f_c, *Pa*	The major principal stress of the Mohr stress circle that is tangential to the yield locus when the minor principal stress is zero (*Figure 4*).	
Wall friction angle	Φ', degrees	The arctan of the wall friction coefficient (*Figure 7*).	
Wall friction coefficient	μ_w	The ratio of the wall shear stress to the wall normal stress at a given normal stress condition.	
Wall yield locus	N/A	A plot of the wall shear stress versus wall normal stress. Used to determine the wall friction angle for a powder-wall combination (*Figure 7*).	
Yield locus	N/A	A plot of shear stress versus normal stress at failure. The yield locus sometimes is called the instantaneous yield locus to differentiate it from the time yield locus (*Figure 4*).	A powder exhibits a series of yield loci for each different level of consolidation (density).

⟨1064⟩ IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY PROCEDURE

INTRODUCTION

Identification of botanical articles can be achieved by the application of multiple techniques, including macroscopic and microscopic descriptions, DNA analysis, and chemical means. *Identification of Articles of Botanical Origin* ⟨563⟩ provides a detailed discussion of these approaches. Chemical identification typically employs chromatographic or spectroscopic procedures to achieve the identification by fingerprint comparison against that of a Reference Standard, monograph description, or a reference chromatogram. Thin-layer chromatography (TLC) is one of the chromatographic techniques used in *USP* monographs for botanical articles in this way. High-performance thin-layer chromatography (HPTLC) is the most advanced version of TLC (see *Chromatography* ⟨621⟩), and a general analytical procedure for the application of HPTLC to the identification of botanicals is

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described in *High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin* ⟨203⟩. In HPTLC, the stationary phase consists of a uniform, typically 200-µm layer of porous (pore size 60 Å), irregular particles of silica gel with a size between 2 and 10 µm and an average particle size of 5 µm, plus a polymeric binder and a fluorescence indicator (F_{254}) coated onto a support, which is typically a glass plate or aluminum foil. Other stationary phases, such as chemically bonded phases (C8, C18, CN, NH2; DIOL) or microcrystalline cellulose, are also available with and without a fluorescence indicator. Because of the greater separation efficiency of the fine particles in HPTLC, the chromatographic system is miniaturized, using smaller developing chambers and shorter developing distances of 6–8 cm, in comparison to classical TLC, where 12–15 cm are required for best separation. As a consequence, less mobile phase and less time are required for chromatogram development. Another effect of miniaturization is the use of smaller sample volumes and the resulting possibility of analyzing more samples per plate than in classical TLC.

In addition, improved layer quality due to smaller particle size positively affects the signal-to-noise ratio, and therefore affects the detectability of separated sample components. A system suitability test is used to qualify the results. This feature is of great importance for the identification of botanical ingredients, which have an intrinsic natural variability in chemical composition. HPTLC features electronic images of chromatograms that allow convenient visual comparison of results obtained for multiple samples against images of chromatograms generated with reference materials. For purposes of identification, the complexity of botanical ingredients is best represented by fingerprints obtained in multiple detection modes from the same chromatogram, e.g., UV 254 nm and UV 366 nm light without derivatization, and white and UV 366 nm light after derivatization. Because compendial methods are used to determine compliance, variability in the results caused by the analytical method selected should be avoided. Because HPTLC can control the variables within narrow ranges using a rigorously standardized methodology and appropriate equipment, HPTLC is able to increase significantly the reproducibility of a chromatographic result from plate to plate compared to traditional TLC. Following here is a discussion of the variables to be controlled in order to achieve reproducible results in the identification of articles of botanical origin using HPTLC.

VARIABLES OF HPTLC

The plate is an open system affected by environmental factors, which must be controlled carefully. Unlike column chromatography, a closed system where samples are analyzed sequentially, in HPTLC multiple samples can be analyzed in parallel on the same plate. All steps of the planar chromatographic process are independent in time and location (an off-line process). For each of the steps—sample application, chromatogram development, visualization, detection, documentation, and evaluation—numerous parameters can be selected freely. This unique feature adds immense flexibility to the method and makes the planar chromatographic approach complementary to column-based techniques.

During method development, the many choices available for the various parameters can be overwhelming, and depending on the combination selected, there may be multiple results that all meet the respective analytical goal and yet are quite different. In this scenario, the labels "better" or "worse" are often assigned to the parameters according to personal preferences. In a cGMP (current Good Manufacturing Practices)-compliant environment, the reproducibility and integrity of results are of great importance. This is why the individual parameters of the HPTLC technique should be selected and combined thoughtfully in a standardized methodology so that the final result is optimized and can be compared to results obtained by different analysts. It may be important to avoid cumbersome and time-consuming steps, or to avoid the use of hazardous or toxic chemicals. Simplicity and clarity are desirable aspects of practical methods.

Handling of Plates

HPTLC plates are delicate and must be handled with care to avoid damaging the layer. When moving the plate, it should be touched only in the upper part above the region of chromatography. Each plate should be labeled with a soft pencil in the upper right (or left) corner. The expected developing distance can be marked on the right (or left) edge with a short pencil mark that goes down to the glass support. Marking the developing distance across the entire plate should be avoided because it is difficult to judge when the rather-diffuse mobile phase front reaches that line. Aligning the front with a short mark on the edge is easier.

Plates should be stored in a place that is free of fumes and dust with the layer facing down to the stack. Shrink wrap of the package should not be in contact with the layer in order to avoid contamination with volatiles from the foil. Generally, HPTLC plates are ready for use without any pretreatment. Older or improperly stored plates may have accumulated impurities and therefore require precleaning. This is the case when after development, the solvent front (or additional secondary fronts) can be seen under UV 254 nm as an intense, broad dark band across the plate. Precleaning can be achieved by developing the plate in methanol to the upper edge. Subsequently, the plate is dried in a clean oven at 120° for 20 min. For cooling down to room temperature and for storage prior to further use, plates can be kept in an empty desiccator or wrapped in aluminum foil. Precleaning may slightly change the selectivity of a plate. Therefore, it is important to define in a method whether or not precleaned plates are to be used. Precleaning with the mobile phase is usually not a good option because it may be difficult to completely remove all components during the drying step.

Plate Layout and Sample Application

The standard format of the HPTLC plate is 20 × 10 cm (width × height). Other sizes (e.g., 10 × 10 cm) could be used as well, but one should keep in mind that for obtaining reproducible results, each size of plate requires a different chamber to maintain the same geometrical aspects as, for example, a twin-trough chamber would have for the standard plate (see *Chromatogram Development*). The optimum developing distance in HPTLC is 6 cm. Cutting plates to less than 10 cm in height provides no additional advantages.

Exact positioning is essential for proper identification of separated zones. For the application of samples with respect to plate layout (*Figure 1*), the following parameters must be considered.

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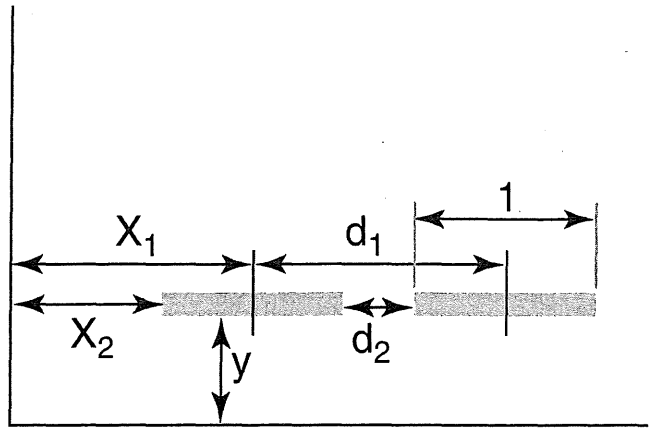

Figure 1. Plate layout.

For proper identification (migration distance/R_F values), all samples must be applied on a (virtual) line parallel to the lower edge of the plate. The distance (y) must be large enough to avoid the sample being immersed in the developing solvent. During development, the velocity of the mobile phase decreases with increasing developing distance. Consequently, the final result depends on the application position relative to the level of developing solvent (*Figure 2*).

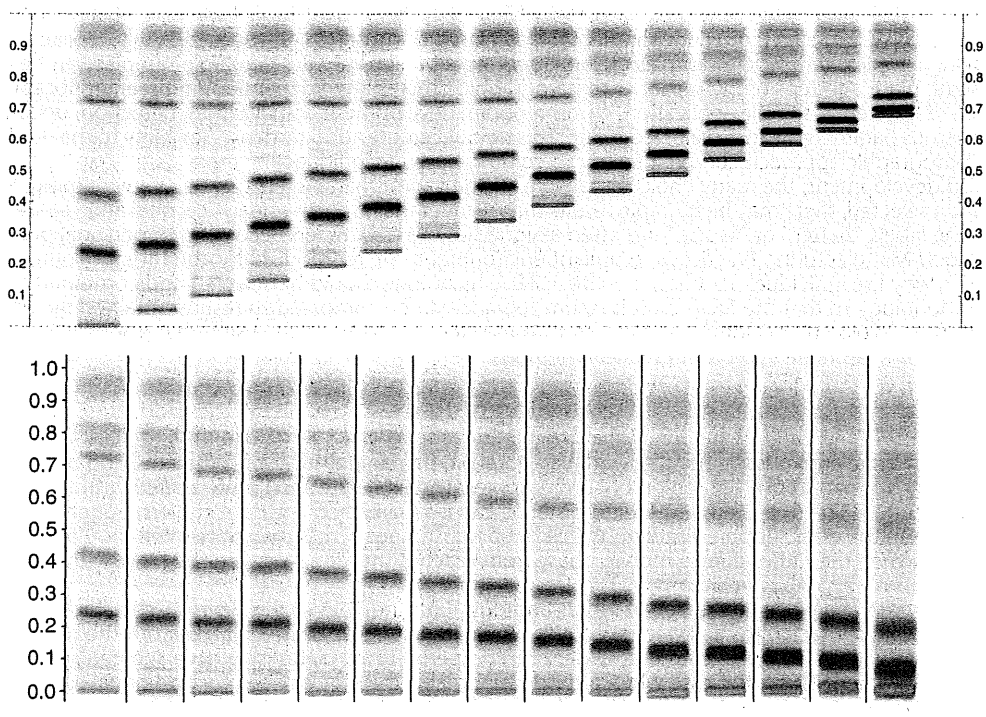

Figure 2. Upper part: Effect of the application position relative to the level of developing solvent (5 mm). Distance from lower edge ranges from 8–50 mm in increments of 3 mm. Lower part: R_F values calculated for the different effective developing distances. Sample is Houttuyniae herba; developing solvent is a combination of ethyl acetate, formic acid, and water (15:1:1, v/v/v); derivatization is Natural Products reagent (NP); detection is UV 366 nm.

For reproducible results, the distance from the lower edge (y) must be kept constant, and the amount of developing solvent placed into the chamber must also be fixed. To minimize solvent consumption, twin-trough chambers are used. The level of developing solvent in such chambers is typically 5 mm. A meniscus is formed on the plate at the immersion line. A sample applied at y = 8 mm will be well above this meniscus.

The distance of the application position from the left and right edges of the plate (x) must be sufficient to avoid the so-called "edge effect" (*Figure 3*). During production, most precoated plates develop a small rim on their edges where the layer is slightly thicker. This causes the mobile phase to advance faster, and samples that are applied directly onto or too close to the rim will migrate unevenly.

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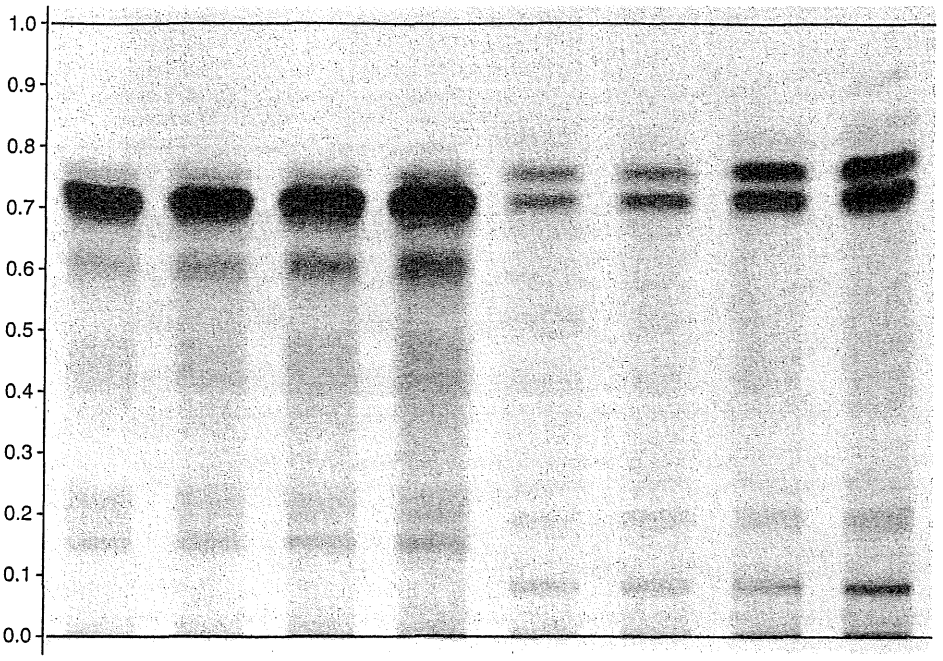

Figure 3. The "edge effect".

The position x_1 is typically called "application position". In order to avoid the edge effect, the length of the applied band (l) has to be taken into account when defining x_2 as the distance from the edge of the plate. A minimum of 15 mm is necessary for x_2. The distance between two samples (d_1) also considers the band length (l). For d_2, a minimum of 3 mm is recommended so that samples do not interfere with each other if larger volumes are applied. The band length (l) must be fixed for each method because it affects the concentration of samples per band if a defined volume of sample solution is applied. The applied quantity (across a defined band length) has an effect on the intensity of the separated zones and may affect whether or not a particular zone of a fingerprint is visible. An argument for selecting shorter bands would be that more samples can be applied on the plate. However, because chromatogram evaluation is primarily performed visually, it has to be noted that a band seems to look "sharper" (more like a band and less oblong) when it is >5 mm (*Figure 4*). A band length of 8 mm is a good compromise also from the perspective that ⟨621⟩ specifies bands of 5–10 mm for HPTLC plates.

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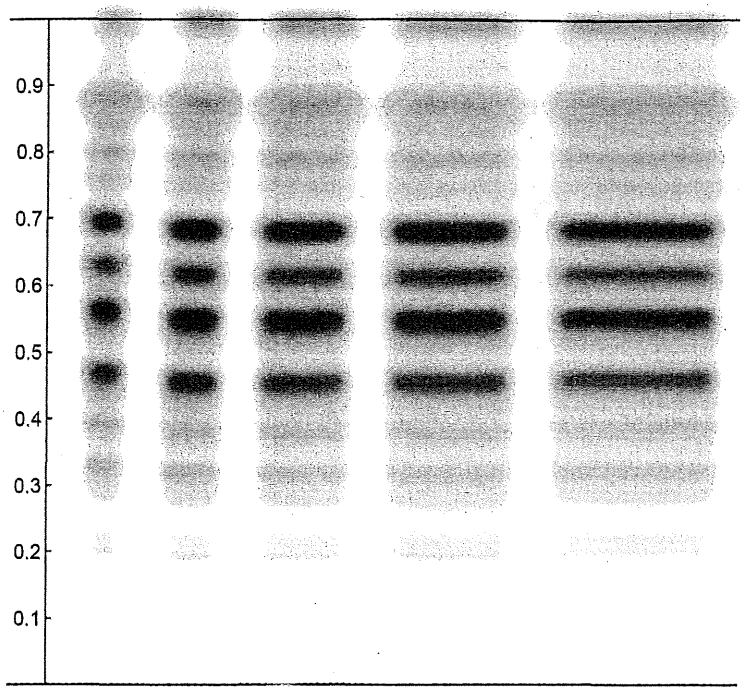

Figure 4. Effect of band length on the visual impression of separation. From left to right, 3 µL/3 mm, 5 µL/5 mm, 8 µL/8 mm, 11 µL/11 mm, and 15 µL/15 mm. Sample is Tiliae flos; developing solvent consists of ethyl acetate, formic acid, water, and methyl ethyl ketone (50:10:10:30, v/v/v/v); derivatization is NP/Polyethylene glycol reagent (PEG); detection is at UV 366 nm.

With the parameters just discussed, the standard plate layout will feature 15 bands of 8-mm length. If fewer samples are to be analyzed, it is recommended to either apply replicates or leave some tracks on the plate blank.

During application, samples are precisely deposited onto the HPTLC plate at defined positions and in defined quantities. Sample solutions can be applied by contact using a capillary or a syringe, or by spraying the sample solution without touching the plate (spray-on technique). During contact application, the sample's solvent can perform circular chromatography (*Figure 5*). Therefore, only small volumes should be applied in one stroke, and if possible, only nonpolar solvents should be used. During spray-on application, the sample's solvent is nebulized and evaporated, creating sharp zones regardless of the polarity of the solvent.

If samples are applied manually, a spotting guide can be used for proper positioning. Marking the application position with a pencil is not a good option, because the layer can be damaged easily.

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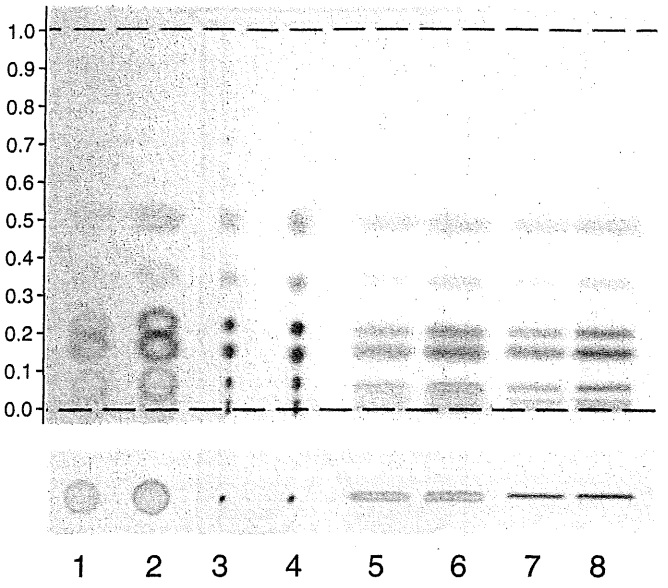

Figure 5. Lower part: Sample application as spot (tracks 1–4) and band (tracks 5–8) using contact application (tracks 1, 2, 5, 6) or spray-on technique (tracks 3, 4, 7, 8); application volumes were 2 µL (tracks 1, 3, 5, 7) or 5 µL (tracks 2, 4, 6, 8); test dye mixture in methanol. Upper part: Chromatography with toluene as mobile phase.

Chromatogram Development

The processes in a chromatographic chamber are highly complex and difficult to describe. Unlike column chromatography, which is assumed to take place in equilibrated chromatographic systems, planar chromatography always begins in a state of nonequilibrium and never reaches equilibrium. The sample has been applied onto the plate, which becomes the stationary phase only when it comes in contact with a liquid in the chromatographic chamber. The advancing mobile phase must be able to dissolve the sample for chromatography to begin. Driven by capillary action, the mobile phase velocity decreases with increasing migration distance because the resistance of the stationary phase against the flow is also increasing.

It can be shown experimentally that the optimum migration distance for mobile phases on HPTLC plates is about 6 cm. Depending on the viscosity of the mobile phase, development takes place between 10 and 20 min. To further move the solvent front just 10 mm will extend the developing time by 5–15 min, while moving an additional 20 mm will increase the developing time by 15–40 min. The contribution of that extra developing distance to improved separation is usually not justifiable, because the diffusion that occurs at decreased velocity of the mobile phase often offsets any gain in distance of separated zones.

Four distinct processes occur in a chromatographic chamber. These processes always take place simultaneously and affect each other (*Figure 6*).

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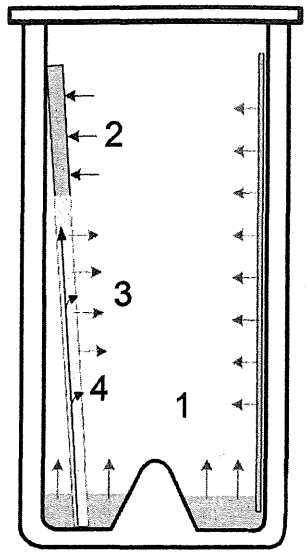

Figure 6. Processes occurring in the chromatographic chamber (twin-trough chamber): (1) chamber saturation; (2) plate preconditioning; (3) evaporation; (4) formation of secondary fronts.

Chamber saturation (*Figure 6*, process 1) is usually established before placement of the plate into the chamber. The developing solvent, which has a known composition, is placed into both troughs of the chamber, and the filter paper in the rear trough is wetted thoroughly. Chamber saturation occurs once the liquid in the tank is in equilibrium with its own vapor, and this is a function of time. After 20 min, the chamber will generally achieve a reproducible degree of saturation.

The plate should be placed into the chamber so that it rests in a vertical position against the front wall while the layer faces the rear wall. The dry portion of the layer absorbs solvent vapor from the gas phase (process 2) in order to achieve adsorptive saturation, which occurs when the surface is covered by a layer of solvent molecules. Generally, this process can lower R_F values and also affect the selectivity of the separation. In the lower portion of the plate, the liquid in the chamber forms the mobile phase. Depending on the degree of saturation, a part of the mobile phase may evaporate off the plate (process 3). Multicomponent mobile phases may be separated by the stationary phase because of different interactions of the constituents. This process is affected by the degree of preconditioning.

In an unsaturated chamber, the situation is quite different. The rear trough does not contain liquid or filter paper. The plate is introduced immediately after the solvent, which does not allow enough time to achieve gas–liquid saturation equilibrium. Preconditioning has little effect, while evaporation and possibly formation of secondary fronts (process 4) dominate the chromatographic process. Both of these processes typically cause alterations in the chromatograms, thereby affecting the separation.

In conclusion, each chamber gives a somewhat different result, depending on the chamber's geometry, the presence or absence of liquid and filter paper in one of the troughs, and the waiting time before introducing the plate. The chromatograms shown in *Figure 7* were obtained with an automatic developing chamber with humidity control. In this example, a saturated chamber (A) gives better separation than an unsaturated chamber (C). Chromatograms are reproducible if all conditions are the same (A) and (B). If the filter paper for saturation is not exchanged but is reused without drying, a completely different result is obtained (D).

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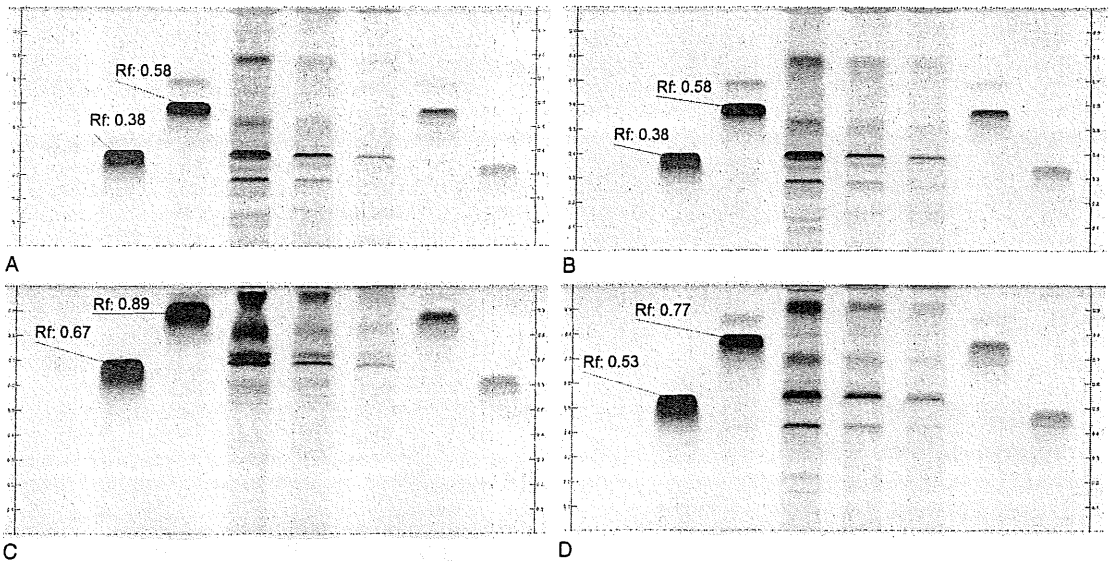

Figure 7. Effects of different chamber configurations at relative humidity 33%: (A) and (B) are saturated, (C) is unsaturated, and (D) is saturated with reused filter paper. Sample is *Lapsana communis*, references are chlorogenic acid (R_F 0.38, Lane 1, Panel A), quercitrine (R_F 0.58, Lane 2, Panel A), developing solvent: water, methanol, glacial acetic acid, and methylene chloride (2:3:8:15, v/v/v/v); derivatization NP/PEG; detection UV 366 nm.

For reproducible results, it is necessary to carefully standardize the development step. Chamber saturation is easy to achieve, as is proper timing. Unsaturated chambers are extremely sensitive to small changes and therefore, from a practical point of view, they should be avoided, even if they can give "better" separation. It is a good practice to keep developing distances constant (at optimum) for all methods, because other parameters, such as selectivity and condition of the gas phase, affect separation to a much greater degree.

Drying

After the mobile phase has advanced the desired distance, the plate must be removed from the chamber and reproducibly dried. This is best achieved by exposing the plate in the vertical position to a flow of cold air. Elevated temperature during drying could cause volatile sample components to diffuse or evaporate from the plate. During the evaporation of the mobile phase, sample components move from deeper areas of the layer to the surface. Uneven drying across the plate may result in different intensities for zones with equal amounts of substances.

Effects of Humidity

Silica gel has an extremely high affinity for water and is an effective drying agent. An HPTLC plate is always at equilibrium with the water vapor in the laboratory atmosphere (relative humidity, RH). Chromatographic results are affected by variation in the amount of water adsorbed onto the silica gel surface. The higher the RH, the more water is adsorbed. In a first approximation, this reduces the "activity" of the stationary phase and causes higher R_F values. Also, because the adsorbed water can become part of the stationary phase, the selectivity of the chromatographic system often changes in response to variation in RH.

Unfortunately, it is nearly impossible to predict what effect a change in RH may have for a given separation (*Figure 8*). Some separations are more affected than others, and sometimes only parts of the chromatogram will change. In addition, there is no ideal activity of the plate that can solve all separation problems, and it is difficult to use defined activity changes of the stationary phase to influence separation in a desired way. The best option is to keep the RH constant at a moderate level (e.g., 33%) and then, try to adjust the selectivity of the mobile phase. One practical exemption is effective "drying off" of the plate using a molecular sieve. Activating a plate by heat (120° for 20 min) is possible, because under these conditions water adsorbed onto the silica gel is effectively removed, but when the plate is cooled down and stored, or during sample application it re-equilibrates to ambient relative humidity.

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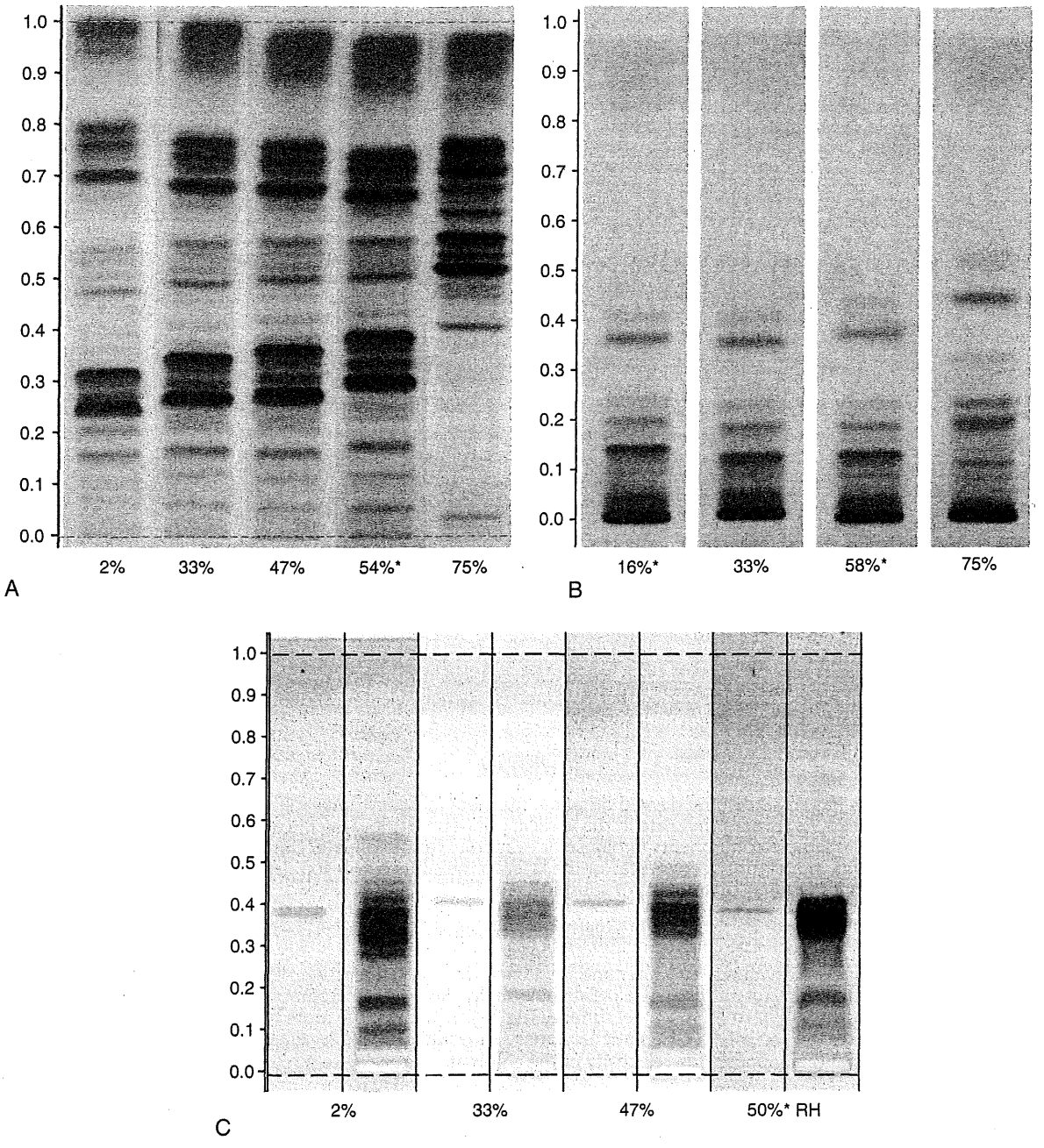

Figure 8. Effects of relative humidity on the separation of different samples. (A) *Hoodia gordonii*, developing solvent: chloroform, methanol, and water (70:30:3, v/v/v); derivatization: anisaldehyde reagent, detection white light. (B) Licorice root, developing solvent: ethyl acetate, formic acid, acetic acid, and water (15:1:1:2, v/v/v/v); derivatization: sulfuric acid reagent, detection white light. (C) Actein/Black cohosh rhizome, developing solvent: toluene, ethyl formate, and formic acid (5:3:2, v/v/v); derivatization: sulfuric acid reagent, detection UV 366 nm. Humidity: *ambient; 2%: molecular sieve; 33%: saturated solution of magnesium chloride ($MgCl_2$); 47%: saturated solution of potassium thiocyanate (KSCN); 75%: saturated solution of sodium chloride (NaCl).

To achieve reproducible results independent of seasonal or regional changes in the RH in various laboratories, it is important to expose the plate to a defined RH before development and with the samples already applied. This will keep the activity and the selectivity of the stationary phase constant. Defined RH can be created in closed containers such as a desiccator over saturated salt solutions. Between 20° and 25°, saturated solutions of magnesium chloride establish 33% RH; of potassium thiocyanate (KSCN), 47% RH; and of sodium chloride (NaCl), 75% RH.

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Derivatization, Detection, and Evaluation

The possibility of convenient chemical derivatization of substances already separated on a plate is an outstanding advantage of planar chromatography. Solutions of derivatizing reagents can be applied onto the plate, either by spraying or immersion. For reproducible results, the amount of reagent of a given concentration applied needs to be defined either by volume (spraying) or by volume, and dwell time (immersion). Most derivatization reactions require a heating step performed either on a plate heater or in a drying oven. The plate should be dried in air before heating to avoid any disruption of the layer resulting from evaporation of residual solvent. Before the plate is heated, the heating device should reach the required temperature. For reproducible results, it is important that the entire derivatization process is strictly timed. This includes any waiting time (e.g., for cooling) before the detection step.

Detection is performed under white light, typically by using a light source above the plate (reflectance) in combination with light from below the plate (transmission). Additional information (typically before any chemical derivatization) can be obtained under UV 254 nm. The F_{254} indicator of the HPTLC plate will emit a green light (blue for F_{254s}). Any substance that absorbs UV 254 nm will be visible as a dark "quenching" zone where the degree of quenching corresponds to the amount of that substance (and its extinction coefficient). Some substances can be excited to fluoresce by UV 366 nm, particularly after chemical derivatization. Such substances are seen as zones of specific colors on the dark background.

HPTLC benefits from digital images taken of the plate in different detection/illumination modes. Such images may sometimes look different from what the analyst's eye is seeing. This is due to some physical limitations, but it is possible to standardize the imaging process so that "true" data are generated. These parameters should always be the same for a given plate regardless of where and by whom the image is taken. The use of image editing software to change certain aspects is not permissible.

In the evaluation step, the analytical data obtained for a sample are compared against specifications of the method. HPTLC for identification of botanical ingredients typically produces fingerprints, i.e., sequences of zones that have specific positions, colors, and intensity. Those fingerprints are compared against data obtained with reference materials. Ideally, comparison is based on images rather than descriptions. Because of the natural variability of botanical materials, there will always be a range associated with the acceptance criteria for a fingerprint to pass the identity test. Fingerprints of different species (adulterants) should have distinguishing features not present in samples that pass the evaluation (*Figure 9*). It is necessary to include a range of botanical reference materials covering different accessions when defining the acceptance criteria for identification. Multiple detection modes are helpful in making decisions.

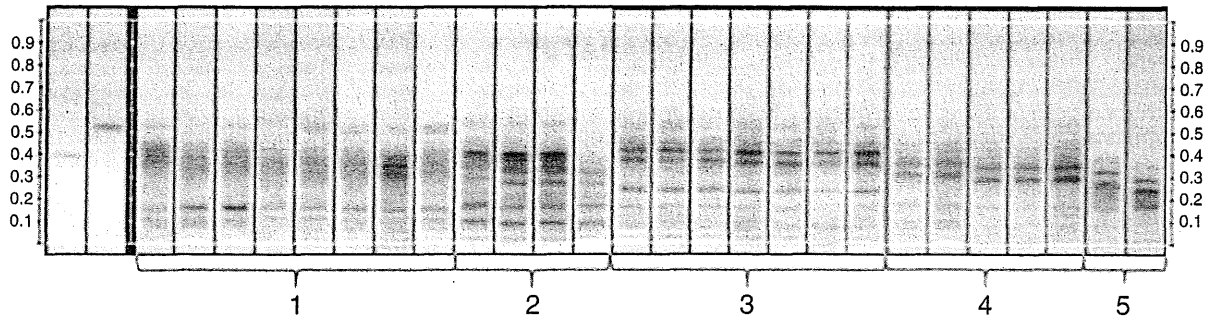

Figure 9. Similarities and differences among multiple samples of related species: (1) *Cimicifuga racemosa*; (2) *Cimicifuga foetida*; (3) *Cimicifuga heracleifolia/dahurica*; (4) *Cimicifuga pachypoda*; (5) *Cimicifuga americana*.

System Suitability

Before evaluating and/or comparing data that originate from different HPTLC plates, each developed plate must be qualified. This is possible with a system suitability test, which is part of the analytical method. On each plate, the analyst selects two or more reference substances that have similar but just separable R_F values under the chromatographic conditions to be used [e.g., chlorogenic acid (blue) and hyperoside (yellow-orange) in chromatographic systems used for flavonoids]. The substances designated to check the system suitability for resolution, position, and colors of the bands may be included in reference standard extracts or another matrix. Description of the resolution position and colors for the key bands of the reference material fingerprint should match the description in the reference within a specified tolerance range. Only when the system suitability requirements have been satisfied can the results obtained with the samples on the same plate be evaluated further to determine compliance. For reference to a standardized procedure for identification of articles of botanical origin controlling the variables described in this chapter, see ⟨203⟩.

CONCLUSIONS

As HPTLC is widely used for botanical identification to monitor the quality of articles of botanical origin on an increasingly globalized level, it seems important to thoroughly standardize the involved analytical procedures. All parameters discussed above must always be selected for each HPTLC analysis. Currently laboratories in different countries can make this selection widely within the framework of the *USP* general chapters; however, guidance about how to achieve specific HPTLC results in a reproducible manner is not available. A standardized procedure as proposed in ⟨203⟩ could fill this gap and serve as basis for harmonization of results obtained in different laboratories.

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⟨1065⟩ ION CHROMATOGRAPHY

INTRODUCTION

Ion chromatography (IC) is a high-performance liquid chromatography (HPLC) instrumental technique used in USP test procedures such as identification tests, assays, and determination of impurities, including limit test and quantitative tests. IC is used to measure anions and cations derived from organic or inorganic molecules, from small molecules to larger biomolecules. This may include, but is not limited to, organic acids, carbohydrates, sugar alcohols, aminoglycosides, amino acids, amines, phosphonates, peptides, aminoglycosides, oligosaccharides, proteins, and glycoproteins.

IC has been applied to all aspects of the manufacturing and disposition of pharmaceutical products, including characterization of active ingredients, excipients, degradation products, impurities, and process streams. Raw materials, intermediates (including media and culture broths), bulk active ingredients, diluents, formulated products, production equipment cleaning solutions, process water, and waste streams may be analyzed using IC.

The majority of IC methods use either anion- or cation-exchange chromatography coupled with suppressed conductivity detection. IC is especially valuable for ionic or ionizable (in the mobile phase) analytes that have little or no native UV absorbance. In addition to suppressed conductivity detection, the ion-exchange separation can be coupled to other detection strategies, including pulsed amperometric detection (PAD), UV/Vis absorbance detection, inductively-coupled plasma mass spectrometric detection (ICPMS), and mass spectrometric detection, providing a wide range of analyte sensitivity and specificity. Ion-exclusion separations expand the range of IC applications to some nonionic analytes (e.g., alcohols) and provide a different selectivity for some analytes that can also be separated by ion exchange. The wide dynamic range of the majority of the IC detection methods makes IC applicable to the quantification of trace contaminants as well as major product components in the same run. IC typically uses dilute acids, alkalis, buffers, or salt solutions as the mobile phase, reducing solvent cost and simplifying disposal logistics. In most cases, the effluent can be disposed of after appropriate neutralization and, when necessary, after dilution with water.

IC is typically performed at or near ambient temperature. As with other forms of HPLC, IC separations are based on varying capacity factors and typically follow the Knox equation. IC is a technique often complementary to reversed-phase and normal-phase HPLC as well as atomic absorption and inductively-coupled plasma techniques in pharmaceutical analysis.

APPARATUS

IC instruments can be either conventional HPLC instruments or instruments specially designed to only perform IC. What type of instrument is chosen depends on the application. Typical components of an IC instrument may include an autosampler, a high pressure pump, a sample loop of suitable size, a guard column, an analytical column, an optional suppressor if conductivity detection is used, a flow-through detector, and a data system (*Figure 1*).

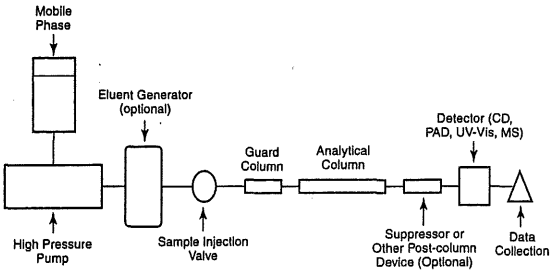

Figure 1. Components of a typical IC system illustrated schematically; CD = conductivity detector, PAD = pulsed amperometric detector, and MS = mass spectrometric detector. [NOTE—Mobile phase is water when the optional *Eluent Generator* is used.]

Because mobile phases generally consist of dilute acids, alkalis, or salt solutions, the components in contact with the mobile phase and the sample are typically made from inert materials, such as polyetheretherketone (PEEK). A conventional HPLC system can be used provided that its components are compatible with the mobile phase and injected sample solutions, though a metal-free system should be used for trace metal analysis. Following suitable preparation, if needed, the sample is introduced via the injection valve. Because IC uses a predominantly ionic mobile phase, a suppressor is typically necessary prior to conductometric detection when high sensitivity is needed, although nonsuppressed conductometric detection has been successfully used in pharmaceutical analysis.

Mobile Phases

Nearly all IC separations require acids or bases diluted in high-purity water, generally with resistivity greater than 18 megohms-cm to prepare the mobile phases. As in every chromatographic method the sensitivity dictates the detection method, and the selectivity dictates the mobile phase and column selection.

For suppressed conductivity detection, the bases and acids used are suppressed to water or weakly dissociated species. If the analytes are anions, the mobile phase bases used as counterions by suppressed conductivity detection are sodium or potassium

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hydroxide, sodium carbonate, sodium bicarbonate, and less often sodium tetraborate. If the analytes are cations, the mobile phase acids used as counterions by suppressed conductivity detection are methanesulfonic acid, sulfuric acid, and less often hydrochloric acid. When determining low ion concentrations, appropriate trapping technology should be used to purify the mobile phase.

When the detection is based on UV or visible absorbance, then a wide variety of salt solutions may be used to prepare the mobile phase, including phthalic acid and *p*-hydroxybenzoic acid for the determination of anions, and methanesulfonic acid for the determination of cations.

Amperometric detection uses either a strong acid or base solution as the mobile phase, although some methods can use a salt solution near neutral pH or an acid or base solution containing a salt. When using alkaline mobile phases, carbon dioxide/carbonate contamination can be minimized through the use of high purity reagents and storage under nitrogen or helium headspace. Most IC-MS methods use the same mobile phases used for suppressed conductivity, though volatile amines and volatile salt solutions have also been used. IC methods that use an ion-exclusion column use solutions of strong acids such as sulfuric acid as mobile phases. Organic solvents commonly used for HPLC are sometimes used to prepare the mobile phase, typically at NMT 20% concentration. This addition is usually made to either modify selectivity or to enhance solubility of sample components that might otherwise contaminate the stationary phase.

Also, protein molecules may get a different surface charge distribution according to their structure and the pH of the mobile phase. In those cases, the pH, gradient of pH, ionic strength, or a combination of those parameters proved to be useful in the separation.

Stationary Phases

IC separations rely on ion exchange, and therefore stationary phases for IC are anion or cation exchangers, and less commonly phases containing both functionalities. Stationary phases may be silica-based or polymer-based materials supporting ionic functional groups. However, due to the solubility of silica gel in water, particularly at alkaline pHs, the use of silica-based support is limited when the mobile phase is alkaline. In those cases, a polymeric support for IC is useful over an extended pH range. Most of the phases used today are constructed with highly cross-linked polymers, increasing their compatibility with the organic solvents that sometimes are needed to prepare the mobile phase. Polymeric anion-exchange phases for IC are typically constructed from polystyrene/divinylbenzene, ethylvinylbenzene/divinylbenzene, or polyvinyl alcohol polymeric substrate and polymethacrylate supports, with particles sizes ranging from 4 to 15 µm in diameter either nonporous or with pores up to 2000 Å. To provide functionality to the anion exchanger, the anion-exchange groups are attached to the substrate typically in one of two manners, electrostatically or covalently by condensation polymerization (a.k.a. a step-growth polymerization). Cation-exchange phases for IC use the same polymeric substrates as the anion-exchange phases, but because the mobile phases for cation IC are acidic, silica can also be used. Ion-exclusion chromatography uses a porous strong cation-exchange stationary phase, while ion-pair chromatography typically uses a polymer reversed-phase stationary phase.

Detection

Conductivity detection is the most commonly employed mode of detection in IC, especially for suppressed conductivity detection.

In suppressed conductivity detection, the background conductance of the ionic mobile phase is significantly reduced as it flows through the suppression device (suppressor). For example, when diluted sodium hydroxide (10–50 mM) used as the mobile phase in IC of anions flows through the suppressor, it is converted to water (H_2O), which shows very poor conductivity. Analogous reactions occur using a suppressor for cations, where the acidic mobile phase is converted to water, and the analyte cations are converted to highly conducting hydroxide forms (due to higher equivalent conductance of hydroxide ions compared to other anions).

The reduced background conductance and the enhanced signal due to the ionic species result in a significantly enhanced signal-to-noise ratio for the conductometric detection of ions in suppressed IC. This results in reduced baseline noise while increasing the sensitivity and reproducibility of the analysis. Commonly used suppressors can be classified into two categories. In the first type, the reactions occur across an ion-exchange membrane with the regenerant ions furnished by either a chemical or as products of electrolysis of water. In the second type, the suppression reactions occur in a packed bed of high-exchange capacity resin or monolith material, with regeneration either by a chemical or by electrolysis of water.

PULSED/INTEGRATED AMPEROMETRIC DETECTION (PAD/IPAD)

PAD and IPAD are modes of amperometric detection that apply more than one potential to the working electrode. These detection modes are commonly used for the detection of electroactive species, e.g., organic compounds such as carbohydrates, sugar alcohols, amino acids, amines, and organic sulfur species that can be easily oxidized. Analytes are detected by an oxidative desorption process at the surface of an electrode located in the column effluent stream. PAD uses one potential for detection while IPAD uses multiple potentials. The current generated during the fixed time periods these detection potentials are applied is integrated to yield charge. Following detection, a series of potentials are applied for fixed time periods to clean the electrode surface. Unlike conventional direct current amperometry that suffers from electrode surface fouling, this rapidly repeating sequence of potentials for detection and electrode cleaning, referred to as a waveform, allows detection and removal of the products of redox reactions from the working electrode surface.

UV DETECTION

Direct UV detection is used for inorganic and organic ions that absorb UV light, typically at low wavelengths. These include organic acids, bromide, iodide, nitrate, nitrite, thiosulfate, and cyano-metal complexes. Indirect UV detection uses eluents that strongly absorb in the visible or ultraviolet spectral region. A wavelength is selected where the eluent absorbs but the sample

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ions do not, and then negative peaks proportional to the analyte concentration are detected. In spectral detection after post-column reaction, some analytes are detected after the column effluent is combined with a reagent resulting in the formation of a compound that absorbs light at either a UV or visible wavelength. A classic example is the determination of metal ions, where the metal ions in the column effluent are chelated with 4-(2-pyridylazo)-resorcinol followed by detection at 510 and 530 nm.

MASS SPECTROMETRY (MS)

Typically, analytes are detected after they have first passed through a suppressor to make the resulting effluent compatible with the mass spectrometer. Negative mode electrospray ionization is used for anions while the positive mode is used for cations. The suppressor effluent is sometimes augmented with an organic solvent to improve ionization for increased sensitivity. Certain metal ions can be determined by an ion-exchange separation followed by ICPMS.

SAMPLE PREPARATION

Sample preparation may range from simple sample dissolution or dilution to the proper concentration, often followed by filtration, and in other cases more complex preparations need solid-phase extraction (SPE). If the solution is cloudy and/or contains particulates, then filtration through a syringe filter of 0.45-µm pore size that is suitable for IC is needed. Samples containing a high concentration of ions of the same charge as the target analyte may require a sample pretreatment to selectively remove the high concentration ion.

PROCEDURE

The choice of mobile phase is typically dictated by the choice of column, which in turn is chosen based on the selectivity for the analyte(s) compared to other ions of the same charge known or likely to be present. In situations where the other ions are in high concentration, a column with higher capacity is chosen to prevent column overload. This is especially important for many limit tests, where low concentration of a target analyte is in the presence of a large concentration of another ion of the same charge state. For anion IC, some mobile phases can be prepared from the solid or from commercial concentrates or ready-to-use solutions, e.g., sodium bicarbonate/carbonate. Other mobile phases should be prepared and handled with care, e.g., sodium hydroxide solutions, minimizing air exposure, and prepared from 50% sodium hydroxide solutions. Sodium hydroxide pellets and commercial dilute solutions contain large amounts of carbonate, thus altering the desired composition of the mobile phase. The acid solutions for cation IC are prepared by diluting high-purity concentrated acids. Alternately, carbonate/bicarbonate, hydroxide, and methanesulfonic acid mobile phases can be produced by an eluent generator. Most analyses will require the injection of 5–50 µL of sample solution, but larger volumes may be required for the analysis of low concentration analytes. As in other LC techniques, quantification is made by either internal or external standardization procedures, where the concentration is calculated by interpolation of the sample response into a calibration curve. IC methods are validated according to the recommendations described in *Validation of Compendial Procedures* ⟨1225⟩.

⟨1066⟩ PHYSICAL ENVIRONMENTS THAT PROMOTE SAFE MEDICATION USE

INTRODUCTION

The physical work environment in healthcare settings is one of the most important potential contributors to medication errors. Data on medication errors reported to the United States Pharmacopeia (USP) Medication Error Reporting Program (2008) show that various physical attributes of the workplace affect human performance on the job. These attributes may help or hinder healthcare providers as they strive to deliver safe, high-quality care to every patient. As noted by the Institute of Medicine (IOM) in its landmark 2001 report, *Quality Chasm*, "Health care today harms too frequently and routinely fails to deliver its potential benefits."

This general chapter describes evidence-based standards for creating and maintaining a physical environment that supports and promotes accurate, safe medication use. The process of safe medication use involves multiple aspects, or stages: procurement, prescribing, transcribing, order entry, preparation, dispensing, and administration, as well as monitoring the medication's effects on the patient. A better understanding of these processes can form a solid foundation for improvement, allowing healthcare providers to reach optimal performance within the medication use system in various practice settings. This general chapter focuses on the characteristics of the physical environment that can promote accurate medication use. Standards are provided when justified by evidence and expert opinion, and a glossary of terms used in this general chapter is provided.

ORGANIZATIONAL SUPPORT

The leadership of a healthcare organization typically determines the operations, policies, and procedures that influence safety. The leaders also establish the workplace design and cultural environment, and they have a powerful influence on the espoused safety culture versus the enacted safety culture. For example, the organizational hierarchy can either enhance safety by encouraging the free flow of information and reinforcing safety behaviors, or can discourage and downplay any safety initiatives.

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Two reports are available that document issues related to organizational actions and culture as they affect patient safety and care: The IOM's 1999 report, *To Err is Human*, and the IOM's 2006 report, *Preventing Medication Errors*.

Accreditation agencies such as The Joint Commission (TJC), the National Integrated Accreditation for Healthcare Organizations (NIAHO), and the International Organization for Standardization (ISO) emphasize the roles of the organization and leadership in determining safety standards. TJC defines the critical function of leadership and holds the leaders accountable for their systems and processes, whereas NIAHO and ISO call for management review of both quality and safety by the governing body, with the goal of promoting high-quality, safe healthcare.

Organizational influence can be understood as falling somewhere on a continuum ranging from prescriptive controls, in the form of rules and procedures, to discretionary controls, which may rely solely on the individual's experience, according to James Reason (*Human Error*, 1990). Organizational controls that foster a safe environment should include, at a minimum, the reporting, analysis, and intervention plans necessary to shore up defenses against adverse medication events. Systems analysis of errors should include the organizational influences that ultimately affect the workforce at the unit level.

Leadership that is supportive and empowering, at all levels of the organization, should result in optimal physical work environments that help to prevent medication errors and promote accurate medication use. This approach can improve the performance of all persons involved in the medication use process. An organization that aligns leadership goals and resources to a strategic safety focus based on evidence will create safer systems for medication delivery. Although hospitals have been "designed" for patient care, there is a growing body of research that points to evidence-based design (EBD) for safe delivery of care. Other high-risk industries with a strategic safety emphasis share common characteristics, including the use of robust processes for improvement throughout organizations; this can lead to interventions based on scientific data and evidence.

MEDICATION SAFETY ZONES

In the medication use system, the degree of accuracy and safety accomplished is the end result of many interactions between humans, their physical work environments, and the equipment and technology they use. It is important to focus on areas of the work environment where (1) medications are prescribed; (2) orders are entered into a computer or transcribed onto paper; and (3) medications are prepared/compounded, dispensed, and administered. These work areas are commonly referred to as medication safety zones (MSZ). Examples include the work areas around a medication cart on a nursing unit; any location where prescribing decisions are made; the work space of an automated medication dispensing device; a pharmacy where prescriptions are prepared, inspected, and dispensed; and areas in patients' homes where medications are prepared, consumed, or administered. The patient's bedside in the healthcare facility or home is another important MSZ that presents unique challenges.

The physical environment of any procedure room deserves special attention because of the increased noise levels, variable illumination, and other distractions. In this challenging setting, providers are making critical medication decisions that can influence whether a patient lives or dies. Also, medication safety zones should include the provision for areas that allocate space to incorporate equipment for the safe disposal of sharps (needles and syringes). Overall, in any MSZ, it is important to ensure that appropriate quantities of supplies are maintained; product expiration dates are checked routinely; and temperature-sensitive products are kept in the correct storage conditions.

Principles From Human-Factors Research

The research literature describes several principles that may be useful in planning the physical environment of the MSZ.

IMPORTANCE PRINCIPLE

Within the MSZ, important components should be placed in convenient locations. For example, information systems should be accessible within or near the MSZ so that drug information and patient data (e.g., lab results and vital signs) are readily available. Instructions about equipment function and troubleshooting should be located directly on or near the equipment to provide a quick answer to questions that arise.

FREQUENCY OF USE PRINCIPLE

Supplies and equipment that are used frequently should be easy to find and accessible. This reduces the likelihood that staff will create workarounds, in which suboptimal equipment/supplies are substituted for the recommended items.

FUNCTION PRINCIPLE

Displays, controls, or supplies related to a specific function should be grouped together. For example, syringes, needles, and alcohol swabs could be stored together in one drawer, and IV tubing and connectors that are used to prepare infusions could be placed together on the same shelf.

SEQUENCE OF USE PRINCIPLE

Items should be placed in an order that reflects the sequence of steps needed to perform the task correctly. For example, sterile gloves are in or with sterile dressing kits, and the items are arranged in sequence. This allows personnel to complete tasks quickly and correctly.

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Bedside Workstations

Methods for performing workplace analysis are available. One important aspect is the medication administration areas at the bedside (in healthcare facilities or the patient's home), which should follow the same design as the centralized MSZ. Distractions are an even greater challenge at the bedside, and measures should be taken to minimize them whenever possible. Information and supplies should be kept accessible (according to the human-factor principles already discussed) and placed in an uncluttered area with adequate lighting. Sharps containers should be placed within easy reach for the provider, but out of high-traffic areas. Each bedside work station should be standardized in design, so that information and supplies do not need to be brought along when moving from one patient bed to another.

Lean Production

Maintaining an efficient, effective workplace reduces the likelihood of errors. One efficient approach to workspace design uses lean-production techniques to enhance desirable, value-added activities while eliminating undesirable activities that lead to waste during work processes.

Three lean-production techniques are as follows: (1) *Visual Controls*, i.e., keeping work processes and indicators in view so that personnel can see the status of tasks at a glance; (2) *Streamlined Layout*, i.e., optimizing the sequence of work processes through facility design; and (3) *Point-of-Use Storage*, i.e., storing supplies at the location where they will be used whenever possible so that personnel can perform tasks more efficiently.[1]

Simplification and Standardization

Making changes that simplify and standardize the patient-care environment decreases the cognitive load, reducing the likelihood of slips and lapses during routine tasks by minimizing decision and manipulation time. Standardization can be used for facility and room design, medical equipment, and medication-related functions (e.g., medication delivery, storage of patient-specific medications). Ensuring ready access to clinical information that is specific to the patient (or the drug) is essential for all areas in which personnel implement steps in the medication-use process.

Innovative Solutions

Another approach to optimizing the design of the MSZ is to involve personnel who perform the tasks on a routine basis. Workers may suggest innovative solutions to work station problems. To support innovation, it is advisable to incorporate flexibility into the MSZ design.

Forcing Functions

Constraint and forcing functions are an effective means of preventing errors, particularly for high-risk medications and situations. Forcing functions do not always refer to device design. The simplest of these do not require technology. One of the first forcing functions identified in healthcare was the removal of concentrated potassium from hospital units to eliminate the risk of inadvertent preparation of intravenous solutions with concentrated potassium, an error that has caused a small but stable number of deaths over the years.

Other examples would include sealing neuromuscular blockers in an intubation kit which lowers the chance of a paralyzing agent being administered to a patient without having access to ventilation support. Additionally, packaging an enteral product so it is physically unable to connect to an intravenous tubing luer lock connector would avert a wrong route error, even if the nurse was working in low-light conditions and initially misidentified the intended route for the tubing. The availability of medication safety technology is never a substitute for safe medication practices within an MSZ. Reports have warned of errors that result from ignoring or overriding safety checks, such as smart infusion pump drug libraries and alarms.

Work System Elements

CHARACTERISTICS OF INDIVIDUALS

Characteristics of the individual performing the work include his or her experience level, age, visual and hearing acuity, distractibility, and level of attention. Humans vary in their responses to the physical environment. In an ideal scenario, the physical environment could be modified on an individual basis. In that way, the environment could be adapted to match the needs of the current user, thereby optimizing the accuracy of his/her performance. Alternatively, the environment should be as supportive as possible to as wide a range of capabilities as possible.

SOUND AND NOISE

The Environmental Protection Agency (EPA) recommends peak sound levels of 45 dB during the day and 35 dB at night in hospitals. The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB. The International Noise Council recommends maximums of 45 dB during the day and 20 dB at night for acute care areas. Ear protection is required when workers are exposed to sound levels averaging 90 dB.

[1] Sanders MS, McCormick EJ. *Human Factors in Engineering and Design*. New York: McGraw-Hill; 1993.

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The standard for sound levels in MSZs is set at 45 dB. This is intended to ensure that critical verbal information can be heard accurately. Healthcare providers should be sensitive to their individual need for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods to enhance audibility and learning, for example, the use of a closed room.

Noise is recognized as a serious health hazard to hospitalized patients, and as a source of interference with effective work performance. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels as a factor contributing to stress for nurses is increasingly being documented. In healthcare facilities, sources of noise can range from overhead paging systems, equipment alarms, heating, ventilation, and air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines. Noise has been cited as one obstacle to the effective performance of nurses. An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB. A study of sound levels during shift changes measured 113 dB.

The following sound-related features may affect accuracy when dispensing medication: predictability of the sound; controllability of the sound; type of task (simple vs. complex); multitasking; and distraction due to noise (which may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks). Out of 58 studies, 7 showed that noise improved performance, while 29 showed that it impaired performance. Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research. This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes–Dodson law).

Noise and other sensory interference can be reduced by employing activities, tools, and principles developed by human factors and engineering experts; many of these principles are already being used by some healthcare organizations. The effects of these and other design improvements for nursing workspaces on patient outcomes and facility performance are being studied as part of a research project (http://www.healthdesign.org/pebble) sponsored by the Center for Health Design, a nonprofit research and advocacy organization. The project has found decreases in medical errors, as well as reductions in patient transfers, nosocomial infections, patient falls, and medication usage. When permitted by infection control guidelines, reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting) can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. Workers who don't have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

ACTIVITIES AND TASKS PERFORMED

When designing an optimal physical environment that promotes accurate medication use, one should consider the activities and tasks that need to be performed. A poorly designed environment or other adverse conditions in the work area can lead to an unsafe adaptation of the procedures. For example, practitioners who have to listen to the constant barrage of equipment alarms that are not set within appropriate parameters (and are producing too many false alarms) may silence the alarms to reduce the noise. However, this limits the practitioner's ability to monitor the safety of the system. Similar situations occur with visual alarms and computer messages that are often ignored or overridden. In general, activities and tasks should be structured so they can be performed with the least amount of difficulty. If workarounds are detected, they should be investigated as an indication that the procedure or workflow is not suited for the task.

Multi-Tasking

The term "multi-tasking" originated in the computer engineering industry; it is the ability of a person to perform more than one task simultaneously. The human brain is wired differently in every individual; therefore, not everyone can multi-task successfully. The need to multi-task is common in all areas of the healthcare environment, whether on a nursing unit, in a pharmacy, in an operating room, or in a special procedure area. Some people can focus on performing more than one task at a time, whereas others cannot. In the human brain, multi-tasking involves linearly switching back and forth among different activities while actually completing one task at a time.

This can be problematic in healthcare settings, particularly if something unexpected happens in relation to one task while the individual is focused on the other task. These interruptions and distractions can lead to medication errors and other forms of patient harm. Another type of multi-tasking error is related to "mental stacking," that is, when several items are under consideration at the same time. An excessive cognitive workload can lead to unintended consequences, and it is important to note that not all practitioners are able to handle established systems and processes in a particular physical environment.

Pharmacists and nurses are practitioners who typically multi-task. Although most prescriptions are prepared or administered one at a time, this does not mean that multi-tasking cannot occur. The work area should be designed to keep individual prescriptions separate while accommodating multi-tasking. Individuals vary in their abilities to perform sequential tasks versus multi-tasking. Currently there is not enough evidence to evaluate an individual's fitness to perform multi-tasking in healthcare settings, but human factors research in controlled laboratory settings suggests that in the future, it may be possible to assess and augment this skill in healthcare providers.

TOOLS AND TECHNOLOGIES IN THE PHYSICAL ENVIRONMENT

In the healthcare setting, tools and technologies that are used to perform the tasks can also interact with the physical environment to affect the likelihood of medication errors. Therefore, the design of the environment should account for the specific tools and technologies that individuals will use to complete their work. For instance, the physical layout of an operating

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room should accommodate the size, maneuverability, and required spacing of the many devices used during surgery. Tools and technologies should also meet ergonomic design standards.

Another example is that labels on medications are sometimes too small to display instructions for preparation, dispensing, or administration; when this occurs, alternatives should be considered. One alternative is to include Quick Response (QR) codes on the labels, which allow healthcare personnel with a web-enabled device (such as an iPad, iPhone, or Android-powered device) to access prescriptive information and handle the product correctly. QR codes are two-dimensional bar codes that, when read by an imaging device, bring the user directly to the website containing the needed information.

Additional factors that should be considered include equipment malfunction, downtime, and vibration.

Equipment malfunction: Any equipment that malfunctions, is damaged, or is lost should be reported to the supervisor immediately. In the case of malfunction, a decision should be made either to repair or to replace the item as soon as possible. In many cases, the item can be replaced rapidly, but some pieces of equipment take longer to disassemble and reinstall. Personnel should confirm that the equipment is in good working order before and after each use. A routine program of confirming equipment function should be in place.

Equipment downtime: The term "downtime" refers to periods of time when a system or item of equipment is unavailable. The outage, or downtime duration, is the length of time that the equipment fails to perform its primary function. The concept of downtime is commonly applied to computer networks and servers but can also be used in other environments when discussing equipment failures. These failures can occur for various reasons, including damage; design flaws; procedural error, i.e., improper use by humans; engineering issues, i.e., how the equipment is used and deployed; overload occurring when system resources are stressed beyond capacity; environmental problems with support systems such as heating, ventilation, and air conditioning (HVAC) and power; and scheduled downtime for maintenance, upgrades, or expansion. Service agreements are a common strategy for minimizing downtime and outages. Procedures should be in place to address downtime.

Equipment vibration: Vibration is a mechanical phenomenon in which oscillations occur around an equilibrium point. Although vibration may be desirable, usually it is undesirable, wasting energy and creating unwanted noise. As examples, the vibrational motions of engines, electric motors, or any mechanical device are typically unwanted and may result from imbalances in the rotating parts, uneven friction, imperfect meshing of gear teeth, and other factors. Careful design and proper installation can usually minimize unwanted vibrations.

DISTRACTIONS AND DISTRACTIBILITY INDEX

The healthcare work environment is replete with distractions and interruptions that can adversely affect work performance and lead to medication errors. Architects, engineers, and other professionals who play a role in workplace design have a responsibility to be educated about, and keenly aware of, this issue so they can design safe, ergonomically ideal workspaces. This approach should have a significant beneficial impact by counteracting the negative effects of interruptions and distractions on patient care. In general, MSZs should be located in areas where the potential for distraction and interruption is minimized. Nurses frequently cite distractions and interruptions as contributing to the incidence of medication errors. Distraction from competing tasks is likely to impair performance in several ways, such as sensory/perceptual interference (e.g., the nurse doesn't hear the alarm because a coworker interrupts with a question), cognitive cost of switching tasks (the nurse responds to an alarm more slowly because it takes time to reorient to the alarmed task after a coworker's question), or prospective memory failure (when returning to the task after the interruption, the nurse omits a step because of forgetting where he/she left off).

In addition, interruptions and distractions have been linked to higher rates of prescription dispensing errors in an ambulatory pharmacy. According to the 2008 USP MEDMARX Data Report, distractions continue to play a major role in medication errors, identified as a contributing factor in 45% of all medication errors in hospitals and health systems. However, interruptions and distractions can be prevented or reduced by giving staff the ability to control and manage their exposure to these disturbances. Personnel can be allowed to adjust features of the MSZ to maximize their concentration and attention levels and to optimize their performance. Adjustable features include a work station that is protected from interruptions and distractions, such as a separate medication room, versus a mobile cart with workspace for those that are not adversely affected by distractions. Individuals have different levels of distractibility, and should be sensitive to their own need for a distraction-free work area. Heightened self-awareness of the adverse impact of interruptions and distractions can help minimize problems. Also, workers can be trained in how to avoid interrupting coworkers for non-urgent requests, particularly while their coworkers are performing medication-related tasks. In a pharmacy study, the most frequent source of interruptions was coworkers asking for assistance.

Techniques that may be effective for decreasing interruptions and distractions include visual cues (such as wearing orange safety vests) and physical barriers, such as preparing doses in a medication room. Personnel can also use checklists to focus or refocus their own attention on the task. Scientific research about distractions and interruptions in the physical environment is limited; therefore, additional studies are needed to identify evidence-based corrective actions that will promote safe medication use.

PHYSICAL ENVIRONMENT ELEMENTS

Factors in the physical environment, such as lighting, noise, temperature, layout, and workstation design, interact with human and task-related factors to influence the accuracy of medication use. The remainder of this general chapter focuses on providing guidance on the optimal environment to improve the performance of persons involved in the medication use process.

WORK SYSTEM DESIGN

According to the Systems Engineering Initiative for Patient Safety (SEIPS) model, five work systems—humans, tasks, technology and tools, organization, and environment—interact to affect employee, organization, and patient outcomes. Because these five work systems are closely interrelated, designers should consider all of the systems. Whenever one work

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element changes, there will be implications for the other elements. The entire work system needs to be well designed to optimize performance and ensure positive outcomes. Although this general chapter describes all the work systems, it focuses on recommendations for the physical environment.

Evidence-Based Design

Evidence-based design (EBD) is a field of study that draws upon the most reliable research data in planning the design of the workplace environment and equipment. EBD is closely related to the systematic hierarchy of evidence-based practice, and uses a systematic process of evaluating scientific research as the basis for design decisions that enhance human performance and reduce stress in the complex environment of healthcare. EBD intersects and combines the domains of ergonomics, human factors, usability, and cognitive psychology to arrive at the best possible work environment.

EBD principles are available to inform environmental design choices. In 2008, Ulrich and associates published a review of the research literature on evidence-based healthcare design, concluding that "The evidence indicates that well-designed physical settings play an important role in making hospitals safer and more healing for patients and better places for staff to work." For example, major advances such as reduced infection rates have been linked to the design and location of sinks for hand washing, proper ventilation, and suitable materials for surfaces.

Application of EBD principles to equipment design is just as essential as applying these principles to the work environment. The use of EBD for intravenous pumps, feeding tubes, and patient-controlled analgesic devices has been described in recent years. Research continues to support the use of standardized fonts and labels for drug identification, as well as EBD for medication storage areas. In each decision regarding the complex healthcare work environment, stakeholders should draw upon the most substantial research evidence available and use it as a major guiding factor. Objects such as furniture, cabinetry, and fixtures should be selected after careful consideration of EBD goals and research. For example, it is ideal if furniture can be configured to create a sense of privacy and minimize visual and auditory distractions during medication transcription, preparation, dispensing, and administration. Furniture should be adjustable to meet a worker's ergonomic needs. In addition, surface contamination can be reduced by selecting nonporous surface materials with no joints or seams, allowing for ease of cleaning.

CHALLENGES IN THE PHYSICAL ENVIRONMENT

Ordering and Transcribing

Errors in ordering medications or transcribing medication orders are well documented in the research literature. Because errors made during these tasks can directly endanger patient safety, ordering and transcribing should be treated as high-risk activities within the healthcare environment. Manual methods of ordering and transcribing are dependent on human performance. Errors may result when the reader misinterprets handwriting flaws, abbreviations, or decimal points, or when there is frank misreading of handwriting. Faxed orders involve additional concerns, because legibility may be poor after transmission and printing.

Computerized systems for ordering, known as computerized physician order entry (CPOE), and transcription of orders by electronic means are not completely protected from the possibility of error. For example, electronic healthcare records have been cited for failures in usability and visual display, and may lack the customization necessary to order for specific populations, such as pediatric patients. Although the electronic transmission of data is considered more secure from errors of interpretation, the addition of decision support in CPOE has not been established as an evidence-based intervention that assures safety. At all times, applications of technology should undergo the same degree of scrutiny as other methods to prevent medication errors.

Preparation and Compounding

The preparation/compounding work area should be designed, arranged, and maintained to facilitate high-quality compounding that meets the standards of *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩. The area for sterile drug preparation should be separate from the area for nonsterile drug preparation (see ⟨795⟩ and ⟨797⟩). Traffic in the preparation/compounding area should be kept to a minimum, with only authorized individuals allowed access. Lighting, temperature, and ventilation should be optimal to prevent decomposition of active pharmaceutical ingredients (APIs), excipients, and drug products. These measures can also ensure a workplace where personnel are not distracted by physical discomfort.

Humidity should be monitored and controlled, because drugs tend to degrade in the presence of moisture. Because of its effect on drug stability and integrity, the humidity in compounding and storage areas is second in importance only to temperature (see ⟨795⟩ and ⟨797⟩). A hygrometer should be placed on an interior wall, but not near an air-handling return, hot plate, or door entrance/exit, so that it will provide a representative reading of the relative humidity in the compounding area or storage facility.

The materials used for the floor, walls, shelving, cabinets, and ceiling should not retain dust, odors, or residues from the compounding activities. Also, the area should be free of dust-collecting overhangs (e.g., ceiling pipes, hanging light fixtures, and ledges). The actual work area should be level, smooth, impervious (free of cracks and crevices), and non-shedding, and the shelving and cabinets should be easy to clean.

Hazardous drugs should be prepared, stored, and handled by appropriately trained personnel under conditions that protect the healthcare workers and others who may come in contact with these drugs. Disposal of all hazardous drug wastes should comply with the applicable federal and state regulations. Any workers who perform routine custodial waste removal and cleaning activities in the storage and preparation areas for hazardous drugs should be trained in appropriate procedures to protect themselves and prevent contamination of the physical environment. This training should include procedures to be followed in the event of a spill.

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For sterile compounding, the primary engineering control (PEC) unit should be placed in a location that will minimize noise and avoid conditions that could adversely affect its operation. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC systems can disrupt the unidirectional airflow in open-faced workbenches. The PEC should be placed out of the traffic flow and in a position that will avoid disruption from the HVAC system and room cross-drafts. In general, all equipment should be selected and installed to create optimal working conditions, with minimal noise and sufficient lighting, so that personnel can perform accurate, high-quality work (see ⟨797⟩).

Dispensing

There are many potential distractions in dispensing areas of pharmacies and other areas where medications are dispensed. These distractions may include ringing telephones and interruptions from patients, other staff, visitors, and others. Distractions should be minimized or eliminated to the greatest extent possible. Although dispensing functions often require multi-tasking, it is optimal that only one prescription or medication order be prepared, processed, and checked at a time. The final check should be done by a pharmacist or an authorized, licensed professional in an area that is free of distractions. Additional points include the following:

- Proper illumination is necessary because the labels on some containers have very small or lightly colored print.
- Noise levels should be kept to a minimum (see *Table 2*), which may require moving noisy equipment to a different room, or at least using a partition for separation.
- Odors in the pharmacy setting should be controlled by using appropriate exhaust systems, which should be maintained properly and monitored periodically. Pharmacies contain a combination of odors resulting from the storage, preparation, and dispensing of the drugs themselves; odors also emanate from other items such as disinfecting agents.
- Temperature should be maintained at appropriate levels for the comfort of pharmacy personnel and for the stability of the pharmaceuticals.
- The pharmacy workspace should be designed to enhance workflow efficiency and to maintain safety throughout the entire dispensing process.

Administration

Some critically important MSZs for nurses include the medication preparation and administration areas. Information should be readily available in a user-friendly format that will facilitate the practitioner's synthesis of facts and data. Access to medication-related information should be efficient, with materials and records available at the proper sites. For example, a nurse may need both drug information and patient-specific data to make a decision about drug administration; therefore, these two sources should be near each other to support fact finding and/or decision making. The various information components within the space should be arranged according to specific principles that decrease distractions when seeking information and making decisions. Nurses also need to be aware of additional factors when administering medications, e.g., illumination, clutter, odor, noise levels, and temperature.

The Home Environment

The home healthcare environment differs from hospitals and other institutional environments in some important ways. For example, clinicians recognize that the homecare setting is the private domain of the patient. Thus the care they provide to each patient should be unique and individualized, based largely on that home setting. There may be situational variables in the patient's home that present risks to the patient and are difficult or impossible for the clinician to eliminate. For instance, hospitals have environmental safety departments to monitor air quality, as well as designers/engineers to ensure that the heights of stair risers are safe, but home healthcare providers are not likely to have the training or resources needed to assess and ameliorate such risks in the patient's home.

Yet the patient and caregiver still can promote a safe home environment by establishing proper storage, organization, and accessibility of medications, as well as adequate lighting in areas where drugs are administered. Several other physical elements also should be assessed to facilitate safe handling and administration of medications. There should be reliable telephone service to the home, an adequate area to prepare and administer medications (e.g., parenteral infusions), and intact, safe electrical outlets for any required equipment. All medicines should be stored in ways that facilitate both medication safety and compliance. The storage locations should be dry and cool. Medications needing refrigeration should be stored in areas where they will not freeze. All medications, whether prescription or over-the-counter, should be kept out of reach of children, pets, or mentally altered/impaired adults.

PHYSICAL ENVIRONMENT FACTORS

Sensory interference resulting from extreme temperatures, noise, poor lighting, glare-producing surfaces, interruptions, or clutter can adversely affect the working memory and job performance of healthcare practitioners. The guidelines described here for the physical environment should be applied to MSZs.

Illumination

Proper illumination levels can improve both accuracy and efficiency of performance. Prescription-filling accuracy improved significantly, from 96.2% to 97.4%, when lighting levels in a busy outpatient pharmacy were increased from 450 to 1460 lux (45–146 fc). One study found that pharmacists who rated lighting levels as at least adequate detected 38% more errors when filling prescriptions. In addition, as visual fatigue increases over a shift, increased light is needed. Pharmacists using task lights

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to increase illumination had a 10.7% reduction in product verification errors. A study of luminance in homes, offices, and public places found lower levels than recommended for reading, and also found that performance was related to age. Efforts should be made to prevent medication errors caused directly or indirectly by low lighting. For example, one incident report showed that poor lighting contributed to improperly connected patient-controlled analgesia (PCA) administration tubing, causing medication to run onto the floor, resulting in uncontrolled patient pain. Low lighting contributed to difficulty in seeing that the tubing was not connected properly. A study of lighting in a retail pharmacy revealed an error in strength and dosage form as dicyclomine 10-mg capsules were used to fill a prescription for 20-mg tablets. The light level at the shelf where the medications were stored was 220 lux (22 fc).

The recommendations described here take into consideration the need to work quickly and accurately during medication handling, the level of task visibility, and the comfort of personnel. Architects and lighting engineers can consult the Illuminating Engineering Society of North America (IESNA) reference "Lighting for Hospitals and Healthcare Facilities" for details about lighting medication areas. It is important to note that the illuminance levels recommended in the IESNA reference are below those listed in this guidance; the latter are higher because of evidence that lighting levels are inversely related to medication errors. Fluorescent cool white deluxe lamps or compact fluorescent lamps are recommended, because they have a color rendering index of 80 or more and are highly efficient compared to incandescent lamps. Providing the recommended color rendering index can help avoid misidentification of medications.

If illumination levels are below recommendations, task lighting is required in areas where critical visual tasks are performed. If task lighting is not available, then workers can cast shadows on the workspace, further reducing lighting levels. Critical tasks include reading handwritten prescriptions and small print on labels and inspecting medication dosage forms. Because individuals perceive lighting levels differently, adjustable 50-watt high-intensity task lights are recommended for situations where personnel encounter difficult-to-read prescriptions and product labels such as unit-dose package labels. Lighting levels are important for key healthcare providers involved in computer order entry (e.g., physicians or pharmacists), prescription filling, inspection, and patient counseling. Illumination levels for computer order entry areas should be at least 750 lux (75 fc). Higher levels [1000 lux (100 fc)] are recommended when handwritten orders are read.

Lighting should be positioned so there is no glare on the computer monitor that may make it difficult to read the screen accurately. Prescription preparation areas, medication inspection stations (for double-checking), and counseling areas should have illumination levels between 900 and 1500 lux (90–150 fc). These standards are all above the minimum of 200 lux (20 fc) for accurate reading of medication labels set by the American Society for Testing and Materials International (ASTM International). An ASTM International standard includes a legibility test requiring that the name and amount of the drug on the label be legible in 20 fc of light at a distance of about 20 inches (500 mm) by a person with 20/20 unaided or corrected vision. Lighting levels should be increased where the work force has an average age beyond 45 years to optimize legibility (general recommendation for treatment of presbyopia). Healthcare providers should also consider having a magnifying glass available to assist in the careful reading of labels with very small print and in situations where low lighting levels are unavoidable. Pharmacists using a magnification lens along with a task light reduced errors in product verification by 22% compared to a control group.

For nurses, key medication-related tasks that require good lighting include medication order review and medication selection, preparation, and administration. These tasks may take place in one or more locations on the nursing unit, such as the nursing station where patient charts are stored, the medication room, or a patient's room. Transitional lighting is recommended for medication areas on nursing stations and other patient care units to avoid dark and bright spots located next to dimly lit areas. Luminance should enable good color rendering (i.e., color rendering index of 80 or more) to assist with proper medication checking. Task lighting can help achieve appropriate levels of lighting and should be included on mobile medication carts (including those used with bar code medication verification systems). Glare on computer monitors should be controlled by ensuring that there are no light reflections that can wash out the screen and make it difficult to read.

Illumination levels for medication rooms located on nursing units should be at least 1000 lux (100 fc) based on the complexity of the task (e.g., reading small type on medication packages) and the need for accuracy and speed. The higher range of the lighting level should be used when the task requires reading small print. Lighting level recommendations are summarized in *Table 1*. Over time, lighting levels can decrease (e.g., by 25% over a 2-year period), so it is important to clean lighting fixtures routinely in order to maintain recommended luminance levels. Lighting levels should be measured on a quarterly basis. Burned out or flickering bulbs should be replaced promptly.

Table 1. Lighting Level Recommendations for Healthcare Settings

	Illumination Level	
Work Area	Lux	Foot-Candle (fc)
Computer order entry	1000	100
Handwritten order processing	1000	100
Medication filling and checking (pharmacy)	900–1500	90–150
Patient counseling (pharmacy)	900–1500	90–150
Sterile compounding and preparation	1000–1500	100–150
Pharmacy medication storeroom	500	50
Medication preparation area (e.g., nursing station)	1000	100
Medication administration work area (e.g., cart surface, patient room)	1000	100

Proper lighting is also essential at the point of care. Attempting to be patient- and family-friendly may run contrary to the necessary lighting conditions for safe medication administration. If practitioners administer medication at night under low

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luminance to avoid disturbing the patient or family, this an unsafe practice. Task or spot lighting should be available so practitioners can visually confirm that they have the correct patient (by reading armband or other identification technology), and so the medication and administration site is not compromised. Compact fluorescent lamps take time to come to the correct lighting level. Therefore, no critical tasks should be performed until the light is working at its rated level.

Sound and Noise Recommendations

For hospitals, the Environmental Protection Agency (EPA) recommends peak sound levels of 45 decibels (dB) during the day and 35 dB at night in hospitals. The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB. The International Noise Council recommends limits of 45 dB during the day and 20 dB at night for acute care areas. Ear protection is required when workers are exposed to sound levels averaging 90 dB. Sound level recommendations are shown in *Table 2*.

Table 2. Peak Sound Level Recommendations

Work Area	Source[a]	Sound Level (dB)
Hospitals (daytime)	EPA	45
Hospitals (nighttime)	EPA	35
Patient room	WHO	35
Acute care areas (daytime)	INC	45
Acute care areas (nighttime)	INC	20
Ear protection required	INC	90 (average)
Medication safety zones	—	50

[a] EPA, Environmental Protection Agency; WHO, World Health Organization; INC, International Noise Council.

The standard for sound levels in MSZs is set at 45 dB, which is intended to ensure that critical verbal information can be heard accurately. However, this will be exceeded in many typical pharmacy dispensing and compounding areas. Healthcare providers should be sensitive to their individual needs for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods (e.g., use of a closed room) to enhance audibility, learning, and privacy.

Excessive noise is recognized as a serious health hazard. Its effect on hospitalized patients and its possible interference with effective work performance by clinicians should be considered. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels are being documented more often as a contributor to stress for nurses. In healthcare facilities, sources of noise can include overhead paging systems, equipment alarms, heating, ventilation, air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines. Noise has been cited as one obstacle to the effective performance of nurses. An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB. A study of sound levels during shift changes measured 113 dB.

Accuracy when dispensing medication can be affected by specific characteristics of the sound/noise, e.g., whether it is predictable and whether it can be controlled. These factors may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks. Out of 58 studies, 7 showed that noise improved performance, while 29 showed that it impaired performance. Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research. This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes–Dodson law).

The adverse effects of noise and other sensory interference can be reduced by using activities, tools, and principles developed by human factors and engineering experts. Many of these principles and tools are already being used by some healthcare organizations. Researchers are studying these and other design characteristics of nursing workspaces to assess their effects on patient outcomes and facility performance; one study is sponsored by the Center for Health Design, a nonprofit research and advocacy organization, and a network of 11 healthcare providers (http://www.healthdesign.org/pebble). The data have shown reductions in medical errors, patient transfers, nosocomial infections, patient falls, and medication usage. Reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting), when permitted by infection control guidelines, can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. In addition, workers who do not have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

Odor

Areas where pharmaceuticals are stored or manipulated tend to have an odor that is characteristic of the respective drugs. These odors often can be minimized by using appropriate air handling/exhaust systems. Indoor environmental quality (IEQ) can be related to chemicals and other sources. The odors found in almost any working environment may result from caulks, sealants, coatings, adhesives, paints, varnishes, stains, wall coverings, cleaning agents, fuels and combustion products, carpeting, vinyl flooring, fabric materials, air fresheners, and other scented products, as well as personal products of employees (e.g., perfumes, shampoos, and others). If these odors are not controlled, IEQ problems can arise, especially if the building's

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ventilation system is improperly designed and/or poorly maintained. IEQ can be improved by installing appropriate exhaust systems to remove the odors emanating from drug products. This may also include "snorkel exhaust" facilities close to where the drug products are manipulated, as well as cabinet-type exhaust hoods. Other measures that may be helpful include establishing perfume-free zones, prohibiting smoking, and not allowing food storage or eating in the immediate area. The practice of introducing pleasant odors in the workplace may or may not be beneficial, but managing the response to odors and irritants is critical to maintaining the health and well-being of workers.

Temperature Controls and Humidity

Temperature control is important for drug stability and for the comfort of personnel. In a room where equipment is present (e.g., laminar air flow hoods), heat tends to be generated, and therefore, appropriate temperature control measures should be designed and implemented.

In some situations such as compounding, it is important to control humidity and thereby minimize static electricity in powders, packaging, and other dosage forms (see ⟨795⟩ and ⟨797⟩). Humidity control is also important for related technologies, such as computers, tablets, and automated dispensing machines. The core temperature of the central processing unit in computers should be between 10° and 32° C, and the ideal humidity index is between 30% and 50%.

Physical Design and Organization of Workspace Assessment

The design of the work environment influences the ability of providers to effectively use information and accurately perform tasks. The height of counters, height of shelving for storage of supplies, and lighting changes in lower drawers and cabinets that decrease visibility of products can contribute to errors if improperly adjusted. The effective integration of adjustable fixtures and appropriate counter heights, with the use of mobile carts, can improve efficiency as well as safety. Work counter clutter or lack of sufficient space to perform key tasks is typically an indicator of disorganization and poor planning. One study found that more dispensing errors occurred when medication storage containers were placed on shelves in a cluttered fashion (<1 inch between distinct drugs). When choosing appropriate furniture, an evidence-based-design furniture checklist may be used to make informed investment decisions and improve healthcare outcomes (see *Table 3* for a complete checklist).

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Table 3. Evidence-Based Design Checklist

Findings	Evidence-Based Design Goals and Features[a]
	1. Reduce surface contamination linked to healthcare associated infections A. Surfaces are easily cleaned, with no surface joints or seams. B. Materials for upholstery are impervious (nonporous). C. Surfaces are nonporous and smooth. 2. Reduce patient falls and associated injuries A. Chair seat height is adjustable. B. Chair has armrests. C. Space beneath the chair supports foot position changes. D. Chair seat posterior tilt angle and seat back recline facilitate patient egress. E. Chairs are sturdy, stable, and cannot be easily tipped over. F. Rolling furniture includes locking rollers or casters. G. Chairs have no sharp or hard edges that can injure patients who fall or trip. 3. Decrease medication errors A. Lighting fixtures should provide 90–150 foot-candle illumination and an adjustable 50-watt high intensity task lamp for furniture with built-in lighting that is used in a medication safety zone. B. Furniture is configurable to create a sense of privacy to minimize visual distractions and interruptions from sound and noise during medication transcription, preparation, dispensing, and administration activities. 4. Improve communication and social support for patients and family members A. Furniture can be configured into small flexible groupings that are easily adjusted to accommodate varying numbers of individuals in a variety of healthcare settings. B. Wide-sized and age variations are supported. C. Acoustic and visual patient privacy are supported. 5. Decrease patient, family member, and staff stress and fatigue A. Materials suggest a link to nature. B. Appearance is attractive and noninstitutional. C. Furniture is tested for safe and comfortable use by all, including morbidly-obese individuals. 6. Improve staff effectiveness, efficiency, and communication A. Furniture is easily adjustable to individual worker's ergonomic needs. B. Design enables care coordination and information sharing. C. Materials are sound absorbing. 7. Improve environmental safety A. Materials do not contain volatile organic compounds (VOCs), such as formaldehyde and benzene. 8. Represent the best investment A. Reflect and reinforce the organizational mission, strategic goals, and brand. B. Integrate new with existing furniture and objects for facility renovations projects. C. Pieces can be flexibly reconfigured and moved to support changing and emerging missions. D. Provide casters or glides to reduce floor damage. E. Check that there are no protuberances that may damage walls; check chair rail heights. F. Manufacturer provides results of safety and durability testing. G. Manufacturer describes the specific evidence that has been used to design the product. H. Manufacturer includes a warranty appropriate to use, such as furniture used all day, every day. I. Replacement parts are available. J. Repair can be done in the healthcare facility. K. Manufacturer or local dealer can assist with furniture repair and refurbishing. L. Environmental services/housekeeping staff can easily maintain furniture. M. A Group Purchasing Organization (GPO) can be used when purchasing furniture.
Findings Scale: Present (+), Absent (–), More Information Needed (?), Not Applicable (N/A).	

[a] Malone EB, Dellinger BA. *Furniture Design Features and Healthcare Outcomes*. The Center for Health Design; 2011 (see https://www.healthdesign.org/chd/research/furniture-design-features-and-healthcare-outcomes).

METHODS FOR ASSESSING THE PHYSICAL ENVIRONMENT

Illumination

An illuminance meter, also referred to as a light level meter or photometer, is an instrument that consists of a photodetector (with a digital or analog display) that measures illuminance in lux or foot-candles (fc). Lighting levels should be evaluated in all MSZs using point illuminance measurements. To do this, the photodetector should be placed in the area where the critical medication task is performed (e.g., medication inspection at a work counter), with the worker standing in a normal working position when the measurement is taken. In medication storage areas, measurements should include light levels at the top, middle, and bottom shelves, because light levels depend on the distance from the lighting source. Photometers are commercially available, or management engineers may be able to provide them, and they should be recalibrated annually.

Illumination levels also can be measured using a smart phone "Light Meter" application (app). These apps are easy to use and inexpensive. They are simply downloaded to a Smartphone, and after clicking on the app, the camera function is pointed to a lighted area/surface and the on/off button is pressed. The device takes measurements and also provides a description of

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the activities for which those light levels are appropriate. The apps can be calibrated and the sensitivity adjusted, with readouts in either lux or fc.

Sound Levels

Sound level meters capable of reading from 30 to 130 decibels on the A scale (dBA) should be used to measure sound levels. The A scale is commonly used when measuring decibels because it most closely represents what the human ear hears in terms of loudness. The meters should be calibrated prior to each use. Measurements are taken while standing in a working position, using the instructions provided in the manual for the specific sound meter. Type 1 or Type 2 meters have acceptable levels of accuracy. Sound levels also can be measured easily and inexpensively by using a Smartphone app. The app is downloaded to a Smartphone, and the readout options are selected. The measurement is activated, and it continues to provide a complete readout of frequency (*x*-axis) and decibel sound pressure level (*y*-axis) in real time.

Odor Detection

Odors can be detected by individuals, but this approach is very subjective. Some odors indicate the presence of a specific chemical entity or moiety. However, appropriate measuring devices for use in a work environment may not be available or practical. Consequently, it may be necessary to select a team of individuals to categorize what is acceptable and what is not acceptable in terms of odors in the work environment. Appropriate HVAC systems may play a role in reducing odors.

Temperature/Humidity Measurement

Temperature is typically measured with digital thermometers, and humidity is measured with hygrometers. In both cases, a recording instrument (e.g., recording thermometer) offers the advantage of documentation.

SUMMARY

As early as the 19th century, Florence Nightingale identified the importance of physical environments for patient healing, but only recently was it established that the physical environment can support the performance of critical tasks by healthcare personnel to protect the patient from iatrogenic harm. Currently, medication errors are recognized as complex, multifactorial, and system dependent, and full scientific understanding of error prevention has proven elusive. However, Nightingale's counsel, "above all, do no harm," compels us to use the most up-to-date evidence for safe practices in healthcare to prevent errors and protect the patient. Aspects and considerations of the physical work environment discussed in this general chapter are not exhaustive, but can serve as a stimulus for the assessment of existing work spaces and for consideration of the latest evidence from human factors science in the design of new facilities. The remaining constituent not discussed in this general chapter is the healthcare provider working in arduous conditions who may, with the best intent, contribute to patient harm through inadvertent actions in substandard conditions. The physical work environment should be structured to support and promote safe delivery of care and prevent harm to patients while also bolstering the practice of healthcare by clinicians.

GLOSSARY OF TERMS

Administering: The preparation, directly prior to use, of a pharmacologic or other therapeutic agent for ingestion, injection, insertion, or application.

Color rendering index (CRI): An expression of how a light source affects the color appearance of objects or humans, compared to how they would appear under a reference light source.

Compounding: The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice.

Constraint: A rule stating under what conditions an action is allowed or prohibited. Constraints are used in designing procedures or tools to prevent unsafe practices.

Crowding: A condition that occurs when multiple workers use the same workspace, adversely affecting the amount of space available for each person and also increasing the negative factors of distractions, interruptions, and noise.

Decibel: A unit used to measure the intensity of a sound by comparing it with a standard level on a logarithmic scale, thereby indicating the degree of loudness. The A scale is commonly used when measuring decibels, because it most closely represents what the human ear perceives in terms of loudness.

Dispensing: The act of providing a medication or prescription order; to fill a prescription.

Distraction: An external stimulus that occurs when a person is engaged in a task or activity that causes a cognitive or emotional disturbance, but does not result in the discontinuation of the activity, such as a telephone ringing or question from a coworker.

Ergonomic design: Arrangement of a workspace to accommodate each individual's capacities and limitations, allowing them to work safely and efficiently. This includes an optimum ambient environment and adjustable furniture.

Forcing function: An aspect of a design that prevents a target action from being performed, or that allows its performance only if another specific action is performed first.

Human factors: The scientific discipline concerned with interactions among humans and other elements of a system, and the profession that applies the theory, principles, data, and methods to design systems that optimize human well-being and overall system performance.

Illumination level: The quantity of light energy reaching an area as measured (in lux or foot-candles) by a photometer with an illuminance sensor; this indicates brightness. A lux is a unit of illuminance, measured in lumens per square meter. A

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foot-candle (fc) is lumens/square foot, and is also commonly measured by light meters. The term candela replaced fc as the International System (SI) measure of luminous intensity and represents 1 lumen/steradium (lm/st).

Interruption: The cessation of productive activity before a task is completed, caused by an externally imposed stimulus.

Lean production: High-quality work output achieved while eliminating waste and decreasing resources used, time spent, and errors.

Medication safety zone: A critical area where medications are prescribed; orders are entered into a computer or transcribed onto paper documents; or medications are prepared/compounded or administered. The characteristics of an optimal physical environment for accurate medication use will apply to MSZs.

Multi-tasking: The ability of human beings to perform multiple tasks simultaneously.

Noise and sound: Noise is defined as an auditory stimulus that bears no informational relationship to the task at hand. Sound is a change in volume that has some informational relationship to the task at hand. A quiet work environment is defined as an area where noise is absent and the worker is free from disturbance.

Override: To neutralize or counteract the action of an automatic control.

Photometer: An instrument for measuring photometric quantities such as illuminance.

Physical environment: The surroundings that can affect one or more human senses.

Workaround: A plan or method used to circumvent a problem (as in computer software) without eliminating it.

Working conditions: A set of factors that include the physical environment, workforce staffing, workflow design, personal/social factors, and organizational characteristics.

Add the following:

▲⟨1071⟩ RAPID MICROBIAL TESTS FOR RELEASE OF STERILE SHORT-LIFE PRODUCTS: A RISK-BASED APPROACH

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USER REQUIREMENT SPECIFICATIONS FOR A RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS
THE CONCEPT OF RISK-BASED MICROBIOLOGICAL MONITORING AND RELEASE TESTING
CRITICAL OPERATING PARAMETERS TO BE USED IN DETERMINING A RISK-BASED RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS
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INTRODUCTION

It is widely recognized that the current growth-based sterility tests with an incubation period of at least 14 days (see *Sterility Tests* ⟨71⟩) are not suitable for products with a short shelf-life or for products prepared for immediate use, which are usually infused into patients before the completion of the test (*1*). These short-life products include compounded sterile preparations (CSPs), positron emission tomographic (PET) products, and cell and gene therapies, which require a new generation of risk-based approaches that include rapid microbial tests. For a general discussion of the factors other than sterility testing that contribute to sterility assurance, see *Sterility Assurance* ⟨1211⟩. It should be noted that as with alternate test methods, the referee test in the event of a dispute is ⟨71⟩.

If a microbial test is conducted, patient safety is best served through the completion of a test that detects microbial contamination prior to product use.

The rapid microbial tests (RMTs) should be risk-based so the stakeholder can select the preferred technology for their intended use and balance user requirement specifications (URS) including time to result, specificity, limit of detection (LOD), sample size, and product attributes. For example, many radiopharmaceuticals, due to the short half-life of radiotracers, would benefit most from a real-time microbial test, while CSPs and cell therapy products, due to their short beyond-use dating, would benefit from an overnight test or at least one that is completed within 48 h.

This general informational chapter discusses the needs of those who manufacture/prepare and test products with a short shelf life and their URS, and includes a brief discussion of some suitable methods for risk-based rapid microbial testing for the release of short shelf-life sterile products (hereafter referred to in this chapter as "short-life products").

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USER REQUIREMENT SPECIFICATIONS FOR A RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

Selection of an appropriate technology for the rapid microbial testing for the release of a short-life product should be a risk-based decision. The URS (*2*) of different technologies include:

- As short as possible time to result, ideally in real time or less than 24 hours, preferably prior to the administration of the product
- Ability to detect, preferably less than 100 colony-forming units (cfu) in the test sample
- Ability to detect a wide range of viable microorganisms in a product
- Sample quantity, i.e., minimum number of articles tested and quantity per container tested that does not consume a large proportion of the available product; whenever feasible, manufacturers should consider assay requirements during process design
- Aseptic test material handling, i.e., closed systems to reduce inadvertent contamination during testing
- Availability of instruments and reagents from multiple vendors
- Availability of reference standards, negative and positive controls, appropriate for technologies that use signals other than the colony-forming unit
- Ease of use/simplicity of test and data interpretation
- Low rates of false positive and false negative results
- A method suitability testing strategy for each specific product
- Improved patient safety arising from:
  - Completion of the test prior to administration
  - Tests that provide progressive monitoring and reports of a detection of sterility test failure
- Ability to identify the detected microorganisms, which may be useful to the clinician administering the products and investigating to determine its source
- Robustness and reliability of equipment and reagents used in the testing
- Sample preparation suitable for both manual and automated methods

THE CONCEPT OF RISK-BASED MICROBIOLOGICAL MONITORING AND RELEASE TESTING

A review of the URS for an RMT shows that some risk-based decisions would need to be made especially in terms of time to results, LOD, sample size, and range of microbes detected to allow the use of such tests prior to administration. The stakeholders should carry out a risk assessment for choosing an RMT in cases where the current compendial sterility test is unsuitable.

Benefits may include, for instance:

- Use of RMT in cases where a substantial risk to patient survival exists when the product is administered too late. One striking example from the clinical literature is that of bloodstream infections that are rapidly progressive infections with mortality rates of up to 40% and in which each day of delay in administrating antibiotics leads to a 10% increase in mortality (*3*). In these cases, patient safety is clearly promoted by the completion of a microbial test prior to the administration.
- Use of growth-based RMTs with continuous reading applied for "negative to date" on risk release because fast growing microorganisms can be detected earlier, and if a failure is detected this would enable the clinician to intervene sooner with the patient. When a contaminated product is detected, the laboratory supervisor could inform the clinician treating the patient and intravascular fluid resuscitation and antibiotic treatment may be initiated to avoid septic shock. A sterility test that progressively monitors and automatically reports any failure will therefore have additional advantages as compared to a single reading at the end of the incubation period.
- Use of non-growth based RMTs with LOD above 1 cfu that is amplified by growth in microbiological media but very fast time to results. The ability to detect contamination, in real time, prior to the administration of the short-life product may be considered more important than detection of a single colony-forming unit in the product. When considering risk to the patient, choice of the RMT should factor in the sensitivity of the assay versus the time to detection. Assays should be reasonably sensitive to detect the presence of a low-level contaminant and should do so in a time frame that allows results to be available before product administration.
- Other advantages of nongrowth-based RMT methods also may include the inability to be affected by antibiotics in the test sample and the detection of culture-negative infectious agents. Some DNA–targeted antibiotics (e.g., polymyxin B and bacitracin) have been shown to inhibit PCR amplification while antibiotics such as penicillin G, chloramphenicol, amphotericin and nalidixic acid do not affect the resolving potential of the reaction. This would be most important to sterile compounding pharmacies producing injectable antibiotic solutions. Method suitability should determine whether any antibiotics in the test sample could affect the assay.

In addition, RMTs or other rapid microbiological methods (RMMs) may be used as in-process controls prior to the final product release sterility test to provide faster information on the effectiveness of microbial controls and the early detection of gross contamination (enabling to investigate and restart production sooner) or probability that a product may fail sterility.

For the risk assessment, one consideration that may be overlooked is the relative risk to the patient based on the volume of the product injected or infused and the site of administration. The greater the volume and the more invasive, the greater the

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risk of blood stream infection for the patient. The risk ranges from a small volume of an intradermal injection to a large volume of an intravenous infusion (see *Table 1*).

Table 1. Typical Volumes by Route of Administration and the Relative Risk Level

Route of Administration	Typical Volume Administered (mL)	Risk Level
Intradermal	≤0.1	Very Low
Subcutaneous	≤1	Low
Intramuscular	≤3	Moderate
Intrathecal	1–10	High
Intravenous push	1–60	Moderate–High
Intravenous piggyback	25–250	High
Intravenous	>250	High

CRITICAL OPERATING PARAMETERS TO BE USED IN DETERMINING A RISK-BASED RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

The estimated operating parameters, i.e., LOD, time to results, and sample size, for the candidate technologies suitable for an RMT are found in *Table 2*.

Table 2. Operational Parameters of the Candidate Rapid Microbial Technologies

Candidate Technology	LOD (cfu)	Time to Result	Sample Size Range (mL)
Gram stain (for comparative purposes only)	10^4–10^5	30 min	0.1
⟨71⟩	Theoretical LOD of 1–3 cfu based on a Poisson distribution	14 days	40–500
Adenosine triphosphate (ATP) bioluminescence	1–10	2–7 days (including pre-enrichment)	1–1000
ATP bioluminescence	10^3	30 min	1–1000
Flow cytometry	10–100	6–8 h (pre-enrichment)	0.1–2
Isothermal micro-calorimetry	10^4	2–7 days	1
Nucleic acid-methods[a]	10–100	2–4 h	0.2–2
Respiration	1–10	Overnight to 7 days	Up to 10
Solid phase cytometry	1–10	2–3 h	1–1000

[a] For these methods, the signal would be in genomic units.

SITUATIONS WHEN ⟨71⟩ IS UNSUITABLE FOR PRODUCT RELEASE TESTING

Sample Size Consideration

The sample size tested may need to be reduced based on either the sample processing capability of the technology or the need to conserve the much-needed product.

The minimum quantity of product used for each medium and the minimum of units to be tested relative to batch size is found in ⟨71⟩, *Table 2* and *Table 3*. It is widely understood that for large pharmaceutical product batch sizes, the amount of units tested are not statistically based and has a reduced capability of detecting low contamination levels in individual product lots. However, with the smaller batch sizes of many short-lived products, this limitation will be reduced because the proportion of products tested relative to the batch size will increase.

This traditional sampling plan is not appropriate for a cell therapy product. For example, if 10 individual 60-mL IV bags of cells were prepared, then 4 bags would be sampled with 20 mL taken from each bag. With this sampling scheme 40% of the batch would be consumed for sterility testing representing not only a large economic loss, but also more importantly, a huge loss of therapeutic product that may prevent administration of a dose adequate to treat the patient. In contrast, if 40 individual 1-mL vials were consumed for the sterility testing of a 40,000-vial batch of an injectable drug product, this would represent only 0.1% of the batch.

Reducing the sample size and the number of units tested will reduce the sensitivity of the sterility test. *Table 3* and *Table 4* illustrate the relative insensitivity of the test for a pharmaceutical product and a CSP.

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Table 3. The Probability That a 20-Unit Sterility Test Passes Given an Increasing Contamination Rate for a Drug Product

	Contaminated Items in the Batch (%)					
	0.1	1	5	10	20	50
p	0.001	0.01	0.05	0.1	0.2	0.5
q	0.999	0.99	0.95	0.9	0.8	0.5
Probability (*p*) of drawing 20 consecutive sterile items	0.98	0.82	0.36	0.12	0.012	<0.00001

Calculated from the following (*4*):

$$p = (1 - p)^{20} = q^{20}$$

p = proportion of contaminated containers in the batch
q = proportion of non-contaminated containers in the batch

Table 4. The Probability That a 6-Unit Sterility Test Passes Given an Increasing Contamination Rate for a CSP

	Contaminated Items in the Batch (%)			
	1	5	10	20
p	0.001	0.01	0.05	0.1
q	0.999	0.99	0.95	0.9
Probability (*p*) of drawing 6 consecutive sterile items	0.995	0.94	0.73	0.53

Alternative sampling plans have been proposed in other compendia. One recommended approach to sterility testing of cell therapy products for a batch size of less than 40 units is found in *European Pharmacopoeia 9.0* chapter *2.6.27 Microbiological Examination of Cell-Based Preparations*.

The contamination test sample size for a cell preparation with a volume between 10 and 1000 mL would be 1% of the total volume; for a cell preparation with a volume between 1 and 10 mL, it would be a sample size of 0.1 mL; for a cell preparation less than 1 mL, the preparation would not be tested. As suggested in 21 CFR 610.12 (*Sterility*), in-process testing using rapid methods may be used in lieu of final product testing when fully justified.

In a similar fashion as cell-therapy preparations, the sample quantity and sampling plan for PET radiopharmaceuticals must also accommodate the limited number of vials (usually one) and the volume of product produced in a batch (usually less than 15 mL). If the batch is comprised of a single container, the sterility test sample size is at least 1% of the total batch volume. For example, if a batch is comprised of 1 vial containing 15 mL, use at least 0.15 mL for purposes of the sterility test. If the batch is comprised of more than one container, use a volume from a single container that represents at least 1% of the total batch volume. If a batch is comprised of 3 vials each containing 25 mL, use at least 0.75 mL from 1 vial for purposes of the sterility test.

Limit of Detection

Within the limitations of preparing inocula from a cell suspension with one or more colony-forming units, growth-based sterility tests can be shown to have at least a theoretical LOD of 1–3 cfu based on a Poisson distribution. Setting an LOD of a single viable cell with all technologies is an unrealistic barrier of entry for any sterility test, especially when the signal is not the colony-forming unit that is amplified by cultural enrichment.

Ability to Detect a Wide Range of Microorganisms

Although all the analytical platforms should have the ability to detect a wide range of bacteria, yeasts, and molds, it is of practical importance to demonstrate that technology chosen for an RMT is capable of detecting microorganisms implicated in sterility test failures, infection outbreaks, and product recalls associated with either CSPs, radiopharmaceuticals, cell therapies, or manufactured pharmaceuticals. This is especially true if the technology, after risk analysis, is shown to improve patient safety with the administration of the products unique to that stakeholder group.

RAPID MICROBIAL TEST METHODS FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

Technologies recommended based on their match to the URS discussed above (*2*) suitable for an RMT are listed alphabetically as follows:

- Adenosine triphosphate (ATP) bioluminescence

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- Flow cytometry
- Isothermal microcalorimetry
- Nucleic acid amplification
- Respiration
- Solid phase cytometry

Brief Descriptions of the Technologies

Each of these candidate advanced analytical platforms is briefly discussed separately and key references are provided. For an overview, see Moldenhauer (*5*).

ADENOSINE TRIPHOSPHATE BIOLUMINESCENCE

This is a well-established technology with luminometers and reagents available from multiple instrument manufacturers. The energy from living cells is stored as ATP and can be measured as light when exposed to luciferase from the American firefly. Each ATP molecule consumed by luciferase produces 1 photon of light. The result detected by a luminometer is typically expressed in relative light units (RLU) and is instrument, reagent, and organism dependent. The ATP content of different microorganisms ranges from 2 to 4 × 10^{-18} mole/cfu for gram-negative bacteria, 5 to 8 × 10^{-18} mole/cfu for gram-positive bacteria, and 300 to 800 × 10^{-18} mole/cfu for fungi (*6*). Given the high signal-to-noise ratio of the measurement and the background ATP in microbiological culture media, the microbiologically relevant instrument detection limit in broth is in the order of 5000 RLU equivalent to approximately 10^3 cfu.

This LOD will detect the presence of microorganisms at levels which are 3–4 logs less within an aliquot of the media than that is required for visual detection of growth in the media. For a rapid microbial test for the release of sterile short-life products, an enrichment culture either in liquid media to reach a threshold ATP level or on a membrane filter on solid media for the formation of microbial colonies could be used with an incubation time of 2–7 days.

FLOW CYTOMETRY

Flow cytometry may be used to detect fluorescently labeled viable microbial cells after an enrichment culture step of 24–48 h (*7*). A labeling reagent consisting of either a fluorogenic substrate or vital stain is used to differentiate viable cells from dead cells and cellular debris. While bacteria are very small and may be hard to distinguish from cell debris, they can be differentiated by size, shape, and fluorescent intensity. Cell viability is indicated by the ability of the intact cell membrane to retain a fluorochrome generated by nonspecific cellular esterase, or by labeling the cell with nucleic acid-specific vital stains. A laser illuminates each cell in the flow stream and the emitted light is detected by a dual photomultiplier array. The signal is digitized and interpreted by discrimination software. The LOD for this technology may be >1 cfu and an enrichment step may be necessary.

ISOTHERMAL MICROCALORIMETRY

Isothermal microcalorimeters monitor enthalpy changes in closed vials (systems) related to microbial metabolic activity and growth. With current instruments, 10^4 active microbial cells can release enough heat to be detected and enrichment is needed for detection (2–7 days to result). Recently its application in pharmaceutical microbiology has been evaluated, although its specific application to release testing of sterile products has not been established yet (*8*).

NUCLEIC ACID AMPLIFICATION

Real-time quantitative polymerase chain reaction (PCR) has the potential to monitor the exponential phase of PCR through 36–48 cycles of amplification using universal primers and probes (termed "pan-bacterial" and "pan-fungal" methods) to estimate the initial quantity of the target DNA, which is in turn proportional to the number of microbial cells in the test sample. Unlike DNA, cellular RNA is turned-over rapidly metabolically and would be a better indicator of viable microorganisms. For example, *Escherichia coli* contains 2 molecules of DNA and 2 × 10^4 molecules of 16S rRNA per cell (*9*). This process is achieved by the conversion of RNA into a complementary copy of DNA (cDNA) by the enzyme reverse transcriptase and the cDNA can be analyzed in real time in either a quantitative (enumeration test) or qualitative assay (sterility test). Alternatively, for DNA-based PCRs, a sample pretreatment with ethidium monoazide or propidium monoazide may also provide the capability to differentiate live from dead microbial cells (*10*,*11*), or free microbial DNA may be removed from a test sample by a centrifugation/washing step and the bacterial pellet used for analysis.

Realistically, an LOD of a single viable cell is probably an insurmountable challenge, especially for a test relying on a DNA/RNA target and universal primers. Another challenge is the differing amounts of genomic material in different microorganisms.

Generally, the LOD ranges from 10 to 1000 viable cells/mL in a sample and, in some reported cases, from 10 to 100 viable cells/mL. Recently it was shown that PCR may actually achieve detection of microorganisms with a limit of 10^2 to 10^3 cfu/mL in a sample containing a high concentration of up to 10^6 mammalian cells/ mL without the need for pre-incubation in microbial growth media (*12*). Adding a growth-based enrichment step for at least 24–48 h and comparing the PCR results before and after cultural enrichment may provide a practical solution for sterility testing. Alternatively, concentration methods could be applied to enrich the sample and reduce the sample volume. As noted by the authors of a recent study of the use of 16S rRNA PCR sterility test for stem cells, with the demonstrated bacterial sensitivity of 10–100 cfu/mL, a test method with a sensitivity of 100 cfu/mL would be suitable to detect clinically significant bacterial contamination of blood and cell products (*13*).

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Direct comparison of growth-based and nucleic acid amplification-based assays is complicated by the fact that nucleic acid amplification-based assays also detect non-viable organisms and are a measure of microbial genome copy number, not colony-forming units. For that reason, the LOD for a nucleic acid amplification-based assay should be defined in terms of genome equivalents per milliliter.

Non-growth based RMTs for the release of sterile short-life products like nucleic acid amplification may have additional advantages due to the following:

- With close to real-time testing, the test will be completed before the short-life products are infused into a patient
- Detection of culture-negative infectious agents
- The test is minimally affected by antibiotics in the test sample, as indicated earlier in the chapter
- The test is less sensitive to background resulting from animal cell lysis (e.g., particles, ATP) as compared to other technologies since specific microbial genes are targeted

RESPIRATION

This broad category ranges from classical respirometers, to gaseous headspace analyzers to automated blood culture systems. Aerobic and anaerobic broth formulations allow for the recovery of most microorganisms responsible for blood stream infections within a 5-day incubation. With some instruments, this includes incubation at both 20°–25° and 30°–35° as in ⟨71⟩. This technology has been successfully extended to sterility testing of cell therapy products cells using a 7-day incubation as an alternative to the compendial sterility test for lot release (*14*).

Other instruments are available to detect and enumerate respiring microorganisms. For example, tunable diode laser absorption spectroscopy (TDLAS) can measure oxygen (O_2) depletion or carbon dioxide (CO_2) increase in closed units containing growing microorganisms in culture medium. The technology was originally developed to monitor gas headspace composition in closed units and also could be used for automatic media fill inspection (*8,15*). The system has gaseous calibration standards and minor adaptations are needed if the system is to be used for sterility testing (e.g., calibrating for higher-volume containers). [NOTE—All the systems of the respiration platform require microbial growth and metabolic activity for detection, i.e., the usual time of 2–7 days to obtain results is required. However, when the results may be progressively monitored to detect a sterility test failure earlier in the incubation period, that is a huge advantage with short-life products as explained in the risk assessment section above.]

SOLID PHASE CYTOMETRY

There are instrument systems based on solid phase cytometry combining fluorescent labeling and solid phase laser scanning to rapidly enumerate viable microorganisms in filterable liquids (*16*). Microorganisms are collected by filtration on 0.45-micron polyester membranes and treated with background and viability stains. The filters are scanned in a cytometer by a high-speed, 488 nM argon laser. Multiple photomultiplier tubes, processed to differentiate between labeled microorganisms and background noise, based on size, shape, and fluorescence intensity, detect fluorescence. The scan is displayed as a map that identifies the position of the fluorescent events that are verified using an epifluorescence microscope with an automated motorized stage to locate the individual events. The system is claimed to detect individual viable microorganisms in 2–3 h. It should be noted that most cell therapy products are non-filterable, so this technology may not be compatible with these types of products.

METHOD SUITABILITY TESTING

The method suitability requirements for a growth-based sterility test are given in ⟨71⟩. The suitability of the test must be demonstrated for each product to be tested. The recovery of the USP challenge organisms at a level less than 100 cfu, in the presence of residual product, is demonstrated as clearly visible growth in the microbiological growth media for either the direct inoculation or membrane filtration method. With signals other than the colony-forming unit derived from laboratory culture (e.g., nucleic acid, ATP, and fluorescent labeling of viable microbial cells), the results from testing actual samples may give results that are not equivalent to that using other technologies. However, method suitability testing will confirm that product residues in the analyte will not inhibit the enzymatic steps associated with the signal generation by the rapid method.

GLOSSARY

Colony-forming unit (cfu): Viable microorganisms capable of growth on solid microbiological culture media forming discrete, visible colonies.

Limit of detection (LOD): The lowest signal representing a viable microorganism that can be routinely detected.

Patient safety: Reduction of morbidity and mortality of the recipients of a contaminated product that is promoted by the completion of a rapid microbial test prior to the administration.

Positron emission tomography (PET): A nuclear medicine functional imaging technique that is used to observe metabolic processes in the body as an aid to the diagnosis of disease. The system detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body.

Risk-based rapid microbial test: A rapid microbial test selected by the stakeholder after a consideration of the LOD, sample size, specificity, and time to result that promotes patient safety by completing the test prior to the administration of a short-lived product to detect contamination, if any.

Specificity: Ability to detect a wide range of different bacteria, yeasts, and molds.

Time to result: Time to complete the microbial test and reach a conclusion about the lack of contamination of the sample test.

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⟨1072⟩ DISINFECTANTS AND ANTISEPTICS

INTRODUCTION

A sound cleaning and sanitization program is needed for controlled environments used in the manufacture of Pharmacopeial articles to prevent the microbial contamination of these articles. Sterile drug products may be contaminated via their pharmaceutical ingredients, process water, packaging components, manufacturing environment, processing equipment, and manufacturing operators. Current Good Manufacturing Practices (cGMPs) emphasize the size, design, construction, and location of buildings and construction materials, and the appropriate material flow to facilitate cleaning, maintenance, and proper operations for the manufacture of drug products. When disinfectants are used in a manufacturing environment, care should be taken to prevent the drug product from becoming contaminated with chemical disinfectants as a result of the inherent toxicity of the disinfectants. The requirements for aseptic processing include readily cleanable floors, walls, and ceilings that have smooth and nonporous surfaces; particulate, temperature, and humidity controls; and cleaning and disinfecting procedures to produce and maintain aseptic conditions. The cleaning and sanitization program should achieve specified cleanliness standards, control microbial contamination of products, and be designed to prevent the chemical contamination of pharmaceutical ingredients, product-contact surfaces and/or equipment, packaging materials, and ultimately the drug products. These principles also apply to nonsterile dosage forms where the microbial contamination is controlled by the selection of appropriate pharmaceutical ingredients, utilities, manufacturing environments, sound equipment cleaning procedures, products especially formulated to control water activity, inclusion of suitable preservatives, and product packaging design.

In addition to disinfectants, antiseptics are used to decontaminate human skin and exposed tissue and may be used by personnel prior to entering the manufacturing area. Chemical sterilants may be used to decontaminate surfaces in manufacturing and sterility testing areas. Furthermore, sterilants may be used for the sterilization of Pharmacopeial articles, and UV irradiation may be used as a surface sanitizer.

This general information chapter will discuss the selection of suitable chemical disinfectants and antiseptics; the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; the application of disinfectants in the sterile pharmaceutical manufacturing area; and regulation and safety considerations. Biofilm formation and its relationship to

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disinfectants are outside the scope of this chapter. Additional information not covered in the chapter may be obtained from standard texts on disinfectants and antiseptics.[1]

DEFINITIONS

Antiseptic—An agent that inhibits or destroys microorganisms on living tissue including skin, oral cavities, and open wounds.

Chemical Disinfectant—A chemical agent used on inanimate surfaces and objects to destroy infectious fungi, viruses, and bacteria, but not necessarily their spores. Sporicidal and antiviral agents may be considered a special class of disinfectants. Disinfectants are often categorized as high-level, intermediate-level, and low-level by medically oriented groups based upon their efficacy against various microorganisms.

Cleaning Agent—An agent for the removal from facility and equipment surfaces of product residues that may inactivate sanitizing agents or harbor microorganisms.

Decontamination—The removal of microorganisms by disinfection or sterilization.

Disinfectant—A chemical or physical agent that destroys or removes vegetative forms of harmful microorganisms when applied to a surface.

Sanitizing Agent—An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

Sporicidal Agent—An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

Sterilant—An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores. Sterilants are liquid or vapor-phase agents.

Microorganisms differ greatly in their resistance to disinfection agents. The order of resistance of clinically significant microorganisms to chemical disinfectants from most to least resistant is listed in *Table 1*.

Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)

Type of Microorganisms	Examples
Bacterial spores	*Bacillus subtilis* and *Clostridium sporogenes*
Mycobacteria	*Mycobacterium tuberculosis*
Nonlipid-coated viruses	Poliovirus and rhinovirus
Fungal spores and vegetative molds and yeast	*Trichophyton, Cryptococcus,* and *Candida* spp.
Vegetative bacteria	*Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella* spp.
Lipid-coated viruses	Herpes simplex virus, hepatitis B virus, and human immunodeficiency virus

CLASSIFICATION OF DISINFECTANTS

Chemical disinfectants are classified by their chemical type. This includes aldehydes, alcohols, halogens, peroxides, quaternary ammonium compounds, and phenolic compounds (see *Table 2*).

Table 2. General Classification of Antiseptics, Disinfectants, and Sporicidal Agents

Chemical Entity	Classification	Example
Aldehydes	Sporicidal agent	2% Glutaraldehyde
Alcohols	General purpose disinfectant, antiseptic, antiviral agent	70% Isopropyl alcohol, 70% alcohol
Chlorine and sodium hypochlorite	Sporicidal agent	0.5% Sodium hypochlorite
Phenolics	General purpose disinfectant	500 µg per g Chlorocresol, 500 µg per g chloroxylenol
Ozone	Sporicidal agent	8% Gas by weight
Hydrogen peroxide	Vapor phase sterilant, liquid sporicidal agent, antiseptic	4 µg per g H_2O_2 vapor, 10%–25% solution, 3% solution
Substituted diguanides	Antiseptic agent	0.5% Chlorhexidine gluconate
Peracetic acid	Liquid sterilant, vapor phase sterilant	0.2% Peracetic acid, 1 µg per g peracetic acid
Ethylene oxide	Vapor-phase sterilant	600 µg per g Ethylene oxide
Quaternary ammonium compounds	General purpose disinfectant, antiseptic	Concentration dependent on application, Benzalkonium chloride
β-Propiolactone	Sporicidal agent	100 µg per g β-Propiolactone

[1] Ascenzi, J.M., Ed. *Handbook of Disinfectants and Antiseptics,* 5th ed.; Marcel Dekker: New York, 1995; Block, S.S., Ed. *Disinfection, Sterilization, and Preservation,* 5th ed.; Lippincott Williams & Wilkins Publishers: Philadelphia, 2000. Russell, A.D.; Hugo, W.B.; Ayliffe, G.A.J., Eds. *Principles and Practices of Disinfection, Preservation and Sterilization,* 3rd ed.; Blackwell Science Inc.: London, 1999.

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The effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) registers chemical disinfectants marketed in the United States and requires manufacturers to supply product information on the use dilution, type of microorganisms killed, and the necessary contact time. Certain liquid chemical sterilizers intended for use on critical or semicritical medical devices are defined and regulated by the U.S. Food and Drug Administration (FDA).

SELECTION OF AN ANTISEPTIC FOR HAND AND SURGICAL SITE DISINFECTION

Hands and surgical sites are disinfected in a hospital setting to reduce the resident flora and to remove transient flora (e.g., *Streptococcus pyogenes*) and methicillin-resistant *S. aureus* and *P. aeruginosa* that have been implicated in hospital-associated infection. Use of antiseptics to disinfect hands has been shown to be more effective than soap and water in reducing the counts of bacteria on the skin; repeated antiseptic use further reduces these counts. These principles may be applied to clean-room operators in the pharmaceutical industry.

Common antiseptics include 4% chlorhexidine, 10% povidone–iodine, 3% hexachlorophene, 70% isopropyl alcohol, and 0.5% chlorhexidine in 95% alcohol.

SELECTION OF A DISINFECTANT FOR USE IN A PHARMACEUTICAL MANUFACTURING ENVIRONMENT

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following points should be considered: the number and types of microorganisms to be controlled; the spectrum of activity of commercially available disinfectants; the claims as a sterilant; the disinfectant or sanitizer supported by the EPA registrations; the concentration, application method, and contact time of the disinfectant; the nature of the surface material being disinfected and its compatibility with the disinfectant; the amount of organic compounds on the surface that may inactivate a disinfectant; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment with repeated application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning agents and other disinfectants; the planned disinfectant rotation; and the steps that need to be taken to avoid the contamination of pharmaceutical products by a disinfectant.[2]

THEORETICAL DISCUSSION OF DISINFECTANT ACTIVITY

Plots of the log of the number of microorganisms per mL surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time.

The rate constant, K, for the disinfection process can be calculated by the formula:

$$(1/t)(\log N_0/N)$$

in which t is the time, in minutes, for the microbial count to be reduced from N_0 to N; N_0 is the initial number of organisms, in cfu per mL; and N is the final number, in cfu per mL, of organisms.

As with a first-order chemical reaction, the same concentration of disinfectant reduces the number of organisms more rapidly at elevated temperatures. This can be expressed as a temperature, T, coefficient per 10° rise in temperature, Q_{10}, calculated by the formula:

$$\text{Time to decontamination at T°/Time to decontamination at T}$$

in which T is T° – 10.

Further evidence that a first-order reaction is an inadequate description of disinfection is that the Q_{10} values for chemical and enzyme reactions are 2 to 3, while the common disinfectants phenol and alcohol have a Q_{10} of 4 and 45, respectively.

Critical to the successful employment of disinfectants is an understanding of the effect of disinfectant concentration on microbial reduction. A plot of the log of the time to reduce the microbial population in a standard inoculum to zero against the log of the disinfectant concentration is a straight line with the slope of the line termed the concentration exponent, n. The relationship can be expressed as follows:

$$n = (\text{log of the kill time at concentration } C_2) - (\text{log of the kill time at concentration } C_1)/(\log C_1 - \log C_2)$$

in which C_1 and C_2 are the higher and lower disinfectant concentrations, respectively.

The wide differences in concentration exponents, n, have practical consequences in picking the use dilution of different disinfectants and in using dilution to neutralize a disinfectant in disinfectant-effectiveness testing and routine microbial monitoring of the manufacturing environment. For example, mercuric chloride has a concentration exponent of 1, so a 3-fold dilution will reduce the disinfectant activity by 3^1 (or by one-third), while phenol with a concentration exponent of 6 will have a

[2] Denny, V.F.; Marsik, F.J. Current Practices in the Use of Disinfectants within the Pharmaceutical Industry. *PDA J. of Pharmaceutical Sci. and Tech.*, **1997**, *51*, (6), 227–228.

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3^6 (or a 729-fold) reduction in disinfectant activity. Disinfectants with a larger concentration exponent or dilution coefficient rapidly lose activity when diluted. The concentration exponents for some disinfectants are listed in *Table 3*.

Table 3. Concentration Exponents of Common Antiseptics, Disinfectants, and Sterilants

Disinfectant	Concentration Exponents
Hydrogen peroxide	0.5
Sodium hypochlorite	0.5
Mercuric chloride	1
Chlorhexidine	2
Formaldehyde	1
Alcohol	9
Phenol	6
Quaternary ammonium compounds	0.8 to 2.5
Aliphatic alcohols	6.0 to 12.7
Phenolic compounds	4 to 9.9

Another important consideration may be the pH of the disinfectant. Many disinfectants are more active in the ionized form, while others are more active in the nonionized form. The degree of ionization will depend on the pK_a of the agent and the pH of the disinfection environment. For example, phenol, with a pK_a of 10, will be more effective at a pH below 7 where it is nonionized.

MECHANISM OF DISINFECTANT ACTIVITY

Table 4 lists the sites and modes of action of some representative disinfectants.

Table 4. Mechanism of Disinfectant Activity Against Microbial Cells

Target	Disinfectant
Cell wall	Formaldehyde, hypochlorite, and glutaraldehyde
Cytoplasmic membrane, action on membrane potential	Anilides and hexachlorophene
Membrane enzymes, action on electron-transport chain	Hexachlorophene
Action on ATP	Chlorhexidine and ethylene oxide
Action on enzymes with –SH groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, and iodine
Action on general membrane permeability	Alcohols, chlorhexidine, and quaternary ammonium compounds
Cell contents, general coagulation	Chlorhexidine, aldehydes, and quaternary ammonium compounds
Ribosomes	Hydrogen peroxide
Nucleic acids	Hypochlorites
Thiol groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, and hypochlorite
Amino groups	Ethylene oxide, glutaraldehyde, and hypochlorite
General oxidation	Hypochlorite

MICROBIAL RESISTANCE TO DISINFECTANTS

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less likely to occur at significant levels, as disinfectants are more powerful biocidal agents than antibiotics. In addition, they are normally applied in high concentrations against low populations of microorganisms usually not growing actively, so the selective pressure for the development of resistance is less profound. However, the most frequently isolated microorganisms from an environmental monitoring program may be periodically subjected to use-dilution testing with the agents used in the disinfection program to confirm their susceptibility, as there are real differences among different species in resistance to the lethal effects of different sanitizers.

DISINFECTANT CHALLENGE TESTING

Under FIFRA, the EPA requires companies that register public health antimicrobial pesticide products including disinfectants, sanitization agents, sporicidal agents, and sterilants to ensure the safety and effectiveness of their products before they are sold or distributed. Companies registering these products must address the chemical composition of their product, include

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toxicology data to document that their product is safe if used as directed on the label, include efficacy data to document their claims of effectiveness against specific organisms and to support the directions for use provided in the labeling, and provide labeling that reflects the required elements for safe and effective use. While these directions provide valuable information, they may not be helpful in terms of the products' use as disinfectants in a manufacturing environment.

In the United States, the official disinfectant testing methods are published by AOAC International[3] and include the Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporicidal Carrier Test. A scientific study submitted for EPA review in support of disinfectant registration must be conducted at a laboratory facility that follows the Good Laboratory Practices (GLP) regulations (21 CFR 58). To demonstrate the efficacy of a disinfectant within a pharmaceutical manufacturing environment, it may be deemed necessary to conduct the following tests: (1) use-dilution tests (screening disinfectants for their efficacy at various concentrations and contact times against a wide range of standard test organisms and environmental isolates); (2) surface challenge tests (using standard test microorganisms and microorganisms that are typical environmental isolates, applying disinfectants to surfaces at the selected use concentration with a specified contact time, and determining the log reduction of the challenge microorganisms); and (3) a statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant. This is considered necessary because critical process steps like disinfection of aseptic processing areas, as required by GMP regulations, need to be validated, and the EPA registration requirements do not address how disinfectants are used in the pharmaceutical, biotechnological, and medical device industries. For the surface challenge tests, the test organisms are enumerated using swabs, surface rinse, or contact plate methods. Neutralizers that inactivate the disinfectants should be included in either the diluent or microbiological media used for microbial enumeration or both. Information on disinfectant neutralization may be found in *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

The disinfectant efficacy test must have realistic acceptance criteria. In practice, sufficient organisms need to be inoculated on a 2-inch × 2-inch square of the surface being decontaminated, i.e., a coupon, to demonstrate at least a 2 (for bacterial spores) to 3 (for vegetative bacteria) log reduction during a predetermined contact time (i.e., 10 minutes over and above the recovery observed with a control disinfectant application). The efficacy of the neutralizers and their ability to recover inoculated microorganisms from the material should be demonstrated during the use-dilution or surface-challenge studies. Points to remember are that disinfectants are less effective against the higher numbers of microorganisms used in laboratory challenge tests than they are against the numbers that are found in clean rooms (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩); that inocula from the log growth phase that are typically employed in laboratory tests are more resistant, with the exception of spores formed during the static phase, than those from a static or dying culture or stressed organisms in the environment; and that microorganisms may be physically removed during actual disinfectant application in the manufacturing area.

Although not all inclusive, typical challenge organisms that may be employed are listed in *Table 5*.

Table 5. Typical Challenge Organisms

AOAC Challenge Organisms	Typical Environmental Isolates
Bactericide: *E. coli,* ATCC 11229; *S. aureus,* ATCC 6538; *P. aeruginosa,* ATCC 15442	Bactericide: *M. luteus, S. epidermidis, Coynebacterium jeikeium, P. vesicularis*
Fungicide: *C. albicans,* ATCC 10231 or 2091; *Penicillium chrysogenum,* ATCC 11709; *A. brasiliensis,* ATCC 16404	Fungicide: *P. chrysogenum, A. brasiliensis*
Sporicide: *B. subtilis,* ATCC 19659	Sporicide: *B. sphaericus, B. thuringiensis*

Because a wide range of different materials of construction are used in clean rooms and other controlled areas, each material needs to be evaluated separately to validate the efficacy of a given disinfectant. *Table 6* contains a list of common materials used in clean room construction.

Table 6. Typical Surfaces to be Decontaminated by Disinfectants in a Pharmaceutical Manufacturing Area

Material	Application
Stainless steel 304L and 316L grades	Work surfaces, filling equipment, and tanks
Glass	Windows and vessels
Plastic, vinyl	Curtains
Plastic, polycarbonate	Insulation coating
Lexan® (plexiglass)	Shields
Epoxyl-coated gypsum	Walls and ceilings
Fiberglass-reinforced plastic	Wall paneling
Tyvek®	Equipment wraps
Terrazzo tiles	Floors

[3] AOAC International Official Methods of Analysis, 15th, 16th, and 17th editions. Arlington, VA.

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DISINFECTANTS IN A CLEANING AND SANITIZATION PROGRAM

The selection of suitable disinfectants and the verification of their effectiveness in surface challenge testing is critical in the development of a cleaning and sanitization program.

Issues associated with the successful implementation of such a program are the development of written procedures, staff training, decisions on disinfectant rotation, institution of application methods and contact times, environmental monitoring to demonstrate efficacy, and personnel safety.

The cGMP 21 CFR 211.67, *Equipment Cleaning and Maintenance,* details the requirements for written procedures for cleaning, maintenance, and sanitization of pharmaceutical manufacturing equipment. These procedures should address the assignment of responsibility, establishment of schedules, details of cleaning operations, protection of clean equipment prior to use, inspection for cleanliness immediately prior to use, and maintenance of cleaning and sanitization records.

Staff involved in disinfection require training in microbiology, industry practices for cleaning and sanitization, safe handling of concentrated disinfectants, the preparation and disposal of disinfectants, and appropriate application methods. It should be emphasized that the preparation of the correct dilutions is critical because many disinfectant failures can be attributed to use of disinfectant solutions that are too dilute. Typically disinfectants used in aseptic processing and filling areas are diluted with Sterile Purified Water, and are prepared aseptically. Alternately, the disinfectant may be diluted with Purified Water, and then sterile filtered to eliminate microorganisms that may potentially persist in a disinfectant. Diluted disinfectants must have an assigned expiration dating justified by effectiveness studies.

The rotation of an effective disinfectant with a sporicide is encouraged. It is prudent to augment the daily use of a bactericidal disinfectant with weekly (or monthly) use of a sporicidal agent. The daily application of sporicidal agents is not generally favored because of their tendency to corrode equipment and because of the potential safety issues with chronic operator exposure. Other disinfection rotation schemes may be supported on the basis of a review of the historical environmental monitoring data. Disinfectants applied on potential product contact surfaces are typically removed with 70% alcohol wipes. The removal of residual disinfectants should be monitored for effectiveness as a precaution against the possibility of product contamination.

The greatest safety concerns are in the handling of concentrated disinfectants and the mixing of incompatible disinfectants. For example, concentrated sodium hypochlorite solutions (at a concentration of more than 5%) are strong oxidants and will decompose on heating, on contact with acids, and under the influence of light, producing toxic and corrosive gases including chlorine. In contrast, dilute solutions (at a concentration of less than 0.5%) are not considered as hazardous. Under no circumstances should disinfectants of different concentrations be mixed. Material Safety Data Sheets for all the disinfectants used in a manufacturing area should be available to personnel handling these agents. Appropriate safety equipment such as face shields, safety glasses, gloves, and uniforms must be issued to personnel handling the disinfectant preparation, and personnel must be trained in the proper use of this equipment. Safety showers and eye wash stations must be situated in the work area where disinfectant solutions are prepared.

⟨1074⟩ EXCIPIENT BIOLOGICAL SAFETY EVALUATION GUIDELINES

INTRODUCTION

This informational chapter presents a scientifically-based approach for the safety assessment of new pharmaceutical excipients (i.e., those excipients that have not been previously used or permitted for use in a pharmaceutical preparation). The guidelines presented herein provide a protocol for developing an adequate database upon which to establish conditions for the safe use of a new excipient intended for use in products administered by various dosage routes. [NOTE—The final section of this chapter, *Definition of Terms*, lists some terms referred to in this chapter.]

An excipient may perform a variety of functionality roles in a pharmaceutical product; but, unlike pharmacologically active drug entities, the excipient displays either no pharmacological activity or very limited and directed activity. Because of these differences between excipients and active drug substances in terms of risk and benefit relationships and expected biological activities, the approaches for safety assessments of excipients and active drug substances will differ. Therefore, it is important to note that the guidelines presented in this informational chapter apply only to the safety assessment of excipients, not to the safety assessment of active drug substances.

These testing guidelines are informational in nature and are intended to be used by professionals having a knowledge of toxicology and associated sciences. It is also intended that the applicable safety test method requirements of the receiving regulatory authority would be used in a proposal for market entry. For example, if a proposal is to be submitted to the U.S. Food and Drug Administration, that agency's safety test requirements would have to be met. These guidelines do not provide specific details regarding test methodology and data interpretation. Test procedures that are generally recognized by experts and by the regulatory agencies should be used. Alternatives to the use of living animals are encouraged wherever these alternative procedures have been validated for the intended purpose and where it is known that the alternative procedure will provide sufficient data upon which to base a safety judgment. It is recommended that the *Guiding Principles on the Use of Animals in Toxicology* of the Society of Toxicology (1996) and, in other countries, the appropriate legal and professional codes, be adhered to in the conduct of all test procedures. All studies must meet the requirements of the appropriate national good laboratory practice guidelines in effect in the country where the studies are being conducted.

In cases of extensive human experience based upon food use, there may be sufficient information to fulfill the requirements of the guidelines for orally-ingested excipients only. In addition, there may be animal-based data, which was developed for other purposes, that may be used to fulfill the testing guidelines requirements. If the data requirements have been met through prior human use experience and pertinent human data have been collected in a scientifically sound manner, there is no need to provide animal data for those endpoints evaluated by prior clinical experience.

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Some dosage routes offer unique toxicological challenges, and the guidelines include provisions for these routes (e.g., inhalation). Also, further explanation is provided regarding numbers of species and other basic information (e.g., two species, one rodent and one nonrodent).

The extent of information required to define a set of baseline data, which constitute a toxicological and chemical database, is dependent upon the intended use of, and duration of, dosing of the candidate excipient material. It is critical that a thorough review of background information be conducted before embarking on a testing regimen. In addition to literature database reviews, information should be obtained regarding the physical and chemical properties of the compound; its manufacturing process (or processes); and product specifications including limits of impurities, potential for pharmacological activity, exposure conditions (i.e., dose, duration, frequency of use, dosage formulation, and route of administration), and potential user population. Also, base toxicity information covering the topics is fundamental. Particular attention should be addressed to the absorption/distribution/metabolism/excretion/pharmacokinetics (ADME/PK) studies because much of the later decision process will be dependent upon these data.

These guidelines provide a mechanism for obtaining sets of baseline data for all candidate excipient materials. The background information and baseline toxicity information alone may support the use of the candidate excipient either in a short half-life product that is not administered in a frequency that results in a residual excipient build-up in body tissue or in a product used only once or twice in a lifetime, such as a diagnostic agent. Additional tests, listed under *Step 4* of the *Safety Assessment Guidelines*, are necessary for candidate excipient material that is to be used in a manner that will result in short- or intermediate-term repeated exposure in humans—that is, a pharmaceutical product that will be administered for less than 10 days or for 30 to 90 consecutive days, respectively. For a candidate excipient material that is intended for use in a pharmaceutical product intended for either intermittent or chronic administration over a long time period, such as a treatment for psoriasis or an insulin preparation, further tests are required. These tests are listed under *Step 7* of the guidelines and in the appropriate section under *Additional Requirements for Specific Exposure Routes*. While providing guidance for consumer safety, some of the required tests are intended to provide information to address occupational safety (e.g., skin and eye irritation).

The guidelines are summarized in *Table 1*. Tests that are required (R) by the guidelines are distinct from those that are recommended conditionally (C). Whether conditional tests are conducted is dependent upon the conditions of use and available biological data. Consideration must also be given to the requirements of the regulatory authorities when making the decision to test.

Table 1. Summary of Excipient Guidelines

	Routes of Exposure for Humans					
Tests	**Oral**	**Mucosal**	**Dermal/ Topical/ Trans-dermal**	**Injectable***	**Inhalation/ Intranasal**	**Ocular**
Baseline Toxicity Data						
Acute Oral Toxicity	R	R	R	R	R	R
Acute Dermal Toxicity	R	R	R	R	R	R
Acute Inhalation Toxicity	C	C	C	C	R	C
Eye Irritation	R	R	R	R	R	R
Skin Irritation	R	R	R	R	R	R
Skin Sensitization	R	R	R	R	R	R
Acute Injectable Toxicity	—	—	—	R	—	—
Application Site Evaluation	—	—	R	R	—	—
Pulmonary Sensitization	—	—	—	—	C	—
Phototoxicity/Photoallergy	R	—	R	R	R	—
Genotoxicity Assays	R	R	R	R	R	R
ADME/PK-Intended Route	R	R	R	R	R	R
28-Day Toxicity (2 Species)-Intended Route	R	R	R	R	R	R
Additional Data: Short- or Intermediate-term Repeated Use						
90-Day Toxicity (Most Appropriate Species)	R	R	R	R	R	R
Embryo-Fetal Toxicol.	R	R	R	R	R	R
Additional Assays	C	C	C	C	C	C
Genotoxicity Assays	R	R	R	R	R	R
Immunosupression Assays	R	C	C	R	C	C
Additional Data: Intermittent Long-term or Chronic Use						
Chronic Toxicity (Rodent, Nonrodent)	C	C	C	C	C	C

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Table 1. Summary of Excipient Guidelines *(continued)*

	Routes of Exposure for Humans					
Tests	Oral	Mucosal	Dermal/ Topical/ Trans-dermal	Injectable*	Inhalation/ Intranasal	Ocular
Reproductive Toxicity	R	R	R	R	R	R
Photocarcinogenicity	C	—	C	C	C	—
Carcinogenicity	C	C	C	C	C	C

R = Required
C = Conditional

* Intravenous, intramuscular, subcutaneous, intrathecal, etc.

SAFETY ASSESSMENT GUIDELINES

Background Information

Before proceeding to the steps under *Data Requirements and Checkpoints*, the following points should be reviewed and defined:

- Review literature information using all appropriate databases
- Define chemical and physical properties
- Define manufacturing process
- Define product specifications, including impurities and residual solvents (see applicable ICH guidelines)
- Estimate exposure conditions (dose, duration, frequency route)
- Define user population
- Assess potential for pharmacologic activity.

At this point evaluate what is known, and develop the initial approach to testing.

Data Requirements and Checkpoints

STEP 1

Toxicity Data (see *Baseline Toxicity Data*)
The toxicity data should take into account the following information:

- Effects of acute exposure by oral and intended routes
- Effects of repeated exposures by intended routes
- Effects of in vitro genotoxicity assays
- ADME/PK by oral or appropriate routes; single or multiple doses.

STEP 2

Depending on results of above, evaluate effects of a single dose in humans.

STEP 3

Checkpoint: Evaluate results of above and proposed exposure conditions and exposed population. The above data might allow use in a single product with a short half-life (e.g., a diagnostic agent).

STEP 4

Gather the following additional data:

- Effects of subchronic exposure in appropriate species and routes
- Embryo-fetal development studies via appropriate route of exposure
- Additional in vitro and in vivo genotoxicity tests.

STEP 5

Depending on results of above, consideration should be given to testing in humans as part of the clinical trials of an active ingredient or as a stand-alone procedure.

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STEP 6

Checkpoint: Evaluate all of above information. Data might allow use in a variety of products intended for short-term, repeated intake (e.g., an antibiotic). If the ADME/PK studies for a noninjectable excipient show no absorption, data may permit using a product for 30 to 90 consecutive days.

STEP 7

Additional data should be obtained for use in a product taken chronically, either daily or intermittently, over a long time period depending on:

- Results of subchronic studies and long-term toxicity in appropriate mammalian nonrodents
- Reproductive toxicity studies
- Other test results and human exposure data and long-term toxicity or carcinogenicity in rodents.

Baseline Toxicity Data

The following data should be taken into account:

- Appropriate acute toxicity by intended dose routes: skin sensitization, approximate lethal dose method, limit test, etc.
- Other appropriate acute toxicity studies: oral toxicity by limit test or approximate lethal dose method, skin irritation, etc.
- ADME/PK: single or multiple doses.
- Genotoxicity: for example, Ames Test, in vitro chromosome aberration test, mammalian cell mutation assay.
- 28-day repeated dosing studies in two species by appropriate routes (one rodent, one mammalian nonrodent): evaluation of injection site or similar considerations might be necessary depending on route of administration.

[NOTE—1. In those cases where intended route restrictions (e.g., volume, concentration) preclude an adequate assessment of the toxicity of the excipient, development of a toxicity profile by other relevant routes may be needed.

2. The comparison of toxicity and ADME/PK data between oral and intended routes is critical at this point because that knowledge may set the direction for future toxicity testing (e.g., reproductive toxicity testing conducted by oral route rather than intended route). In addition, relevant studies using the intended route and anticipated duration of exposure may preclude performance of additional studies.]

Additional Requirements for Specific Exposure Routes

FOR ORAL EXPOSURE

No additional requirements beyond those presented for *Baseline Toxicity Data*.

FOR MUCOSAL EXPOSURE

No additional requirements beyond those presented for *Baseline Toxicity Data*.

FOR DERMAL, TOPICAL, OR TRANSDERMAL EXPOSURE

Baseline Toxicity Data

- *Effects of Acute Exposure by Transdermal Dose Route:* dermal sensitization study for repeat applications
- *Effects of Repeated Exposures by Transdermal Route*
  1. Photoallergy/phototoxicity study
  2. Studies in two species (one rodent, one mammalian nonrodent) by transdermal route.
- *Effects of Subchronic Exposure, Reproductive Toxicity Effects*—Initial toxicity studies may be performed by the IV route to adequately profile the toxicity of the excipient. This will provide an assessment of potential target organs if an adequate amount of the compound cannot be delivered via a transdermal dosage form. This is dependent upon the results from the ADME/PK studies.

Reproductive studies may also be conducted via oral or IV route with demonstration of absorption (oral) and pharmacokinetic comparisons of the chosen route versus transdermal.

Photocarcinogenicity studies may be required and should be considered if data and the proposed use indicate when evaluating materials to be placed on the skin for prolonged periods of time and exposure to UV light is a factor (e.g., sun block). This also applies to oral, parenteral, and inhalation products where skin drug concentrations exceed plasma drug concentrations for a substantial period of time, or where the candidate material would appear to have the potential for photo-activity or has demonstrated photo-activity.

FOR INJECTABLE DOSAGE FORMS

Background Information

1. Define compatibility of the dosage form with blood, if appropriate, based on route of exposure.
2. Define the pH and tonicity of injectable dose form, if appropriate, based on the route of exposure.

Baseline Toxicity Data

- *Effects of Acute Exposure by Intended Injectable Dose Routes*

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1. Include evaluation of injection site irritation in rabbit or dog
2. Include evaluation of rate of administration.

FOR INHALATION OR INTRANASAL EXPOSURE[1]

Baseline Toxicity Data

- *Acute Inhalation Toxicity*—A limit test that would, for example, use the highest achievable concentration in a 4-hour exposure to vapor, aerosol, or solid particulate. Pulmonary sensitization may be performed along with other appropriate studies. If exposure is to be to an aerosol or solid particulate, particulates of appropriate mass median diameter should be generated.
- *Single and Repeated Dose ADME/PK by Inhalation or Intranasal and Oral Routes*
- *28-Day Repeated Dose Inhalation Study in Two Mammalian Species Using Vapor or Particulates of Appropriate Mass Median Diameter:* compare to similar oral toxicity data.

FOR OPHTHALMIC EXPOSURE

Background Information: define pH and osmolarity of topical ocular dose form.
Baseline Toxicity Data

- *Effects of Acute Exposure by Ophthalmic Routes:* cytotoxicity tests (e.g., agar overlay)
- *Effects of Repeated Exposures by Ophthalmic Routes*
  1. Studies in two species (one rodent, one mammalian nonrodent)
  2. Examination of anterior and posterior segments of the eye
  3. Studies on allergenicity potential.

Other Data—Comparison of pharmacokinetic parameters of the route chosen for reproductive studies and the ophthalmic exposure are essential for extrapolation of potential toxicity via the ophthalmic route.

GLOSSARY

Acute: exposure to a test agent within a single, 24-hour period. Doses may be single, multiple or continuous during a 24-hour period.

Subacute: repeated dosing of a test agent for up to 29 days. Daily doses may be single, multiple or continuous during a 24-hour period.

Subchronic: repeated dosing of a test agent for 30 days to 10% of the lifespan of the test species (90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

Chronic: repeated dosing of a test agent for more than 10% of the lifespan of the test species (more than 90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

⟨1078⟩ GOOD MANUFACTURING PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

BACKGROUND

This general information chapter provides guidelines for methods, facilities, and manufacturing controls to be used in the production of pharmaceutical excipients in order to ensure that excipients possess the quality, purity, safety, and suitability for use that they purport to possess. The principles and information in this chapter can be applied to the manufacture of all pharmaceutical excipients (referred to throughout this document as *excipient[s]*) intended for use in human drugs, veterinary drugs, and biologics. It covers the quality management system and the extent of good manufacturing practices (GMP) necessary throughout manufacturing for both batch and continuous processes. It is intended to assist manufacturers as well as auditors in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality and purity that they purport to possess and are suitable for their intended use. The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients for parenteral, ocular, inhalation, and open wound use and those that are sterile and/or pyrogen-free. It does not provide information for all national legal requirements nor does it cover in detail the particular characteristics of every excipient. The quality system standard used as a framework for this chapter is ISO 9001, which is appropriate to manufacturing. Because of the diversity of excipients, some principles in this information chapter may not be applicable to certain products and processes.

This chapter combines the concepts of existing GMP principles from the following sources:

- World Health Organization (WHO) GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC) Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,

[1] When designing studies to evaluate use in products intended for use by the inhalation or intranasal route, consideration should be given to the dosing regimen that will be used by humans. The appropriate study protocol for a product intended for inhalation therapy that will result in prolonged exposures (e.g., several hours per day) may differ from that used to evaluate a product that would result in exposure to several metered doses per day.

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- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients,
- International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, deference to all schemes is becoming necessary. Therefore, relevant portions of the manufacturing concepts are employed throughout this chapter.

The *General Guidance* section provides an overview of the appropriate manufacturing practice criteria applicable to excipient manufacture and the points of application of excipient good manufacturing practices and quality systems. The section also recommends measures to limit contamination of an excipient. Finally, it discusses the relationship of excipients to finished dosage forms. No attempt has been made to include details specific to particular excipients.

The information in *Appendix: Auditing Considerations* sets forth key criteria to aid in the audit of an excipient manufacturing facility.

For a list of terms used in this chapter and their definitions, see *Glossary.*

INTRODUCTION

Purpose and Scope

This chapter defines the extent and point of application of appropriate GMP principles for excipient manufacture and is applicable to the manufacture of excipients intended for use in drug products. It covers the quality management system and the extent of GMP necessary throughout manufacturing for both batch and continuous processes. It is intended to aid both auditors and manufacturers in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality, purity, and safety that they purport to possess and are suitable for their intended use.

The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients

- for parenteral, ocular, inhalation, and open wound use; and
- those that are purported to be sterile and/or pyrogen-free.

In these cases, detailed information pertaining to the intended use of an excipient as provided by the end user can be useful in determining appropriate GMP. This chapter does not address the specific GMP relating to good trade and distribution practices (GTDP).

Principles Adopted

THE CHAPTER AND ITS USE

Pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. Each manufacturer should consider how the chapter might apply to its products and processes (for example, batch versus continuous processes). Because excipients are so diverse, some principles of this chapter may not be applicable to certain products and manufacturing processes.

APPLICATION

The text provides information necessary for the manufacture of excipients but does not provide all the details. It cannot specify national legal requirements or cover particular characteristics of every excipient.

QUALITY SYSTEM STANDARD

The quality management system standard chosen as a framework for this chapter is ISO 9001, which is appropriate for manufacturing facilities. A manufacturer may apply the ISO standard with or without certification; but this possibility, as a business decision, is not discussed in this chapter. However, ISO certification has the benefit of providing assurance to customers that the excipient manufacturer's quality management system has been independently verified.

The headings in this chapter have been aligned with the ISO 9001 clause numbers, because many excipient manufacturers already use that standard as a basis for their quality management system. Additional headings are included as needed to introduce additional guidance on GMP when not covered by current ISO 9001 clauses.

DOCUMENT STRUCTURE

The chapter combines the concepts of existing GMP principles from the following:

- World Health Organization (WHO), GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC), Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients, International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, relevant portions of the manufacturing concepts detailed in these schemes are employed throughout this chapter.

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The *General Guidance* section provides an overview of the GMP criteria applicable to excipient manufacture and the point of application of excipient GMP.

The remaining sections provide guidance on GMP principles and implementation of a quality management system suitable for excipient manufacture. For example, these sections suggest measures to limit excipient contamination. No attempt has been made to include details specific to particular excipients, and individual manufacturers should address these as they apply to their own products and processes.

The Appendixes provide supporting guidance for excipient GMP. *Appendix: Auditing Considerations* describes key criteria to be considered in the audit of an excipient manufacturing facility. *Glossary* provides definitions of terms used in this chapter.

GENERAL GUIDANCE

Pharmaceutical Excipients

Pharmaceutical excipients are substances other than the active pharmaceutical ingredient (API) that have been appropriately evaluated for safety and are intentionally included in a drug delivery system. For example, excipients can do the following:

- aid in the processing of the drug delivery system during its manufacture,
- protect, support, or enhance stability, bioavailability, or patient acceptability,
- assist in product identification, and
- enhance any other attribute of the overall safety, effectiveness, or delivery of the drug during storage or use.

A more complete classification of excipients according to their functions can be found in *USP and NF Excipients, Listed by Category* in the *USP–NF*.

Excipient GMP Implementation

The application of GMP is relevant once it has been determined that a chemical is intended for use as a component of a drug product. Excipient manufacture should be carried out in accordance with the GMP concepts consistent with this chapter. The objective of excipient GMP is to ensure that the manufacture of an excipient results in a consistent material with the desired quality characteristics. The emphasis of GMP for excipients is to ensure product integrity, avoid product contamination, and ensure that records are maintained.

As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product should increase. Manufacturing processes should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in this chapter should be applied and maintained.

Judgment based on risk analysis and a thorough knowledge of the process is required in order to determine from which processing step GMP should be implemented. This is usually well before the final finishing operation and, for example, may be identified using methods such as hazard analysis and critical control point (HACCP), failure mode and effects analysis (FMEA), or a detailed process flow diagram. Consideration should also be given to other factors such as batch versus continuous processing, dedicated versus multipurpose equipment, and open versus closed processes.

QUALITY MANAGEMENT SYSTEM: EXCIPIENT QUALITY SYSTEMS

General Recommendations

The principles outlined in this chapter provide a comprehensive basis for the quality management system used in the manufacture of pharmaceutical excipients. Excipient manufacturers should identify the quality management processes required to ensure excipient quality. Where manufacturing, testing, or other operations that could affect excipient quality are outsourced, the responsibility for quality remains with the excipient manufacturer, and control measures should be defined (see also the subsection *Purchasing Information* in the *Product Realization* section).

Documentation Recommendations

GENERAL

The excipient manufacturer should have a system in place to control documents and data that relate to the requirements of the quality management system.

QUALITY MANUAL

The excipient manufacturer should prepare a quality manual describing the quality management system, the quality policy, and the commitment of the excipient manufacturer to applying the appropriate GMP and quality management standards contained in this chapter. This manual should include the scope of the quality management system, reference to supporting procedures, and a description of the interaction between quality management processes.

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CONTROL OF DOCUMENTS

The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of controlled documents, including documents of external origin that are part of the quality management system.

Procedures used in the manufacture of excipients should be documented, implemented, and maintained. In addition, there should be formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is being used throughout the operational areas and that previous revisions of documents have been removed.

Documents and subsequent changes to documents should be reviewed and approved by designated qualified personnel before issuance to the appropriate areas, as identified in the documents. Documents that affect product quality should be reviewed and approved by the quality unit (see also *Responsibility and Authority* in the section *Responsibility, Authority, and Communication* under *Management Responsibility*).

Controlled documents may include a unique identifier, the date of issue, and a revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. When it is practical, changes and the reasons for the changes should be documented.

Electronic documentation should meet the requirements for the document control system stated above. If electronic signatures are used on documents, they should be controlled to provide security equivalent to that provided by a handwritten signature. Electronic documents and signatures may also have to satisfy local regulatory requirements.

CONTROL OF RECORDS

The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records.

Records should be maintained to demonstrate achievement of the required quality and the effective operation of the quality management system. Records should be legible and identifiable with the product involved. Pertinent subcontractor quality data should be an element of these records.

Entries in records should be clear, indelible, made directly after performing the activity (in the order performed), and signed and dated by the person making the entry. Corrections to entries should be signed and dated, leaving the original entry legible.

Records should be kept for a defined period. This period should be appropriate to the excipient and to its expiry date or reevaluation interval. Records should be stored and maintained in such a manner that they are readily retrievable, in facilities that provide an environment suitable for minimizing deterioration or damage.

CHANGE CONTROL

The excipient manufacturer should establish and maintain procedures to evaluate and approve changes that may affect the quality of the excipient. For example, this may include changes to the following:

- raw materials or packaging and their sources,
- material specifications,
- test methods,
- manufacturing and analytical equipment,
- production processes,
- manufacturing or packaging sites.

A unit with a function that is independent from production (such as regulatory affairs or quality assurance) should have the responsibility and authority for the final approval of changes.

Customers should be notified, and, where applicable, excipient regulatory submissions (for example, for Drug Master Files [DMFs] or Certificates of Suitability to the *European Pharmacopoeia* [CEPs]) should be amended to reflect significant changes from established production and process control procedures that may affect excipient quality (see also *Customer Communication* in the section *Customer-Related Processes* under *Product Realization*).

MANAGEMENT RESPONSIBILITY

Management Commitment

Top management should demonstrate to the organization the importance it places on customer satisfaction and compliance with the appropriate regulations and standards. This should be accomplished through the development of a quality policy and establishment of quality objectives. Progress toward the documented quality objectives should be reviewed at planned intervals.

Customer Focus

It is the responsibility of top management to ensure that customer requirements are determined and met. The excipient manufacturer should permit the customer or its representative to conduct audits of the manufacturer's quality management system, manufacturing processes, buildings, and facilities.

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Quality Policy

Top management should demonstrate its commitment to the corporate quality policy and ensure that it is implemented within the operational unit. The quality policy should support continual improvement of the quality management system. Management should participate in the development of the company's quality policy and provide the resources necessary for its development, maintenance, and deployment.

Planning

QUALITY OBJECTIVES

Top management should set objectives for adherence to GMP to ensure that the excipient manufacturer maintains and improves its performance. Objectives should be deployed throughout the organization and should be measurable and consistent with the quality policy.

QUALITY MANAGEMENT SYSTEM PLANNING

Top management should provide adequate resources to ensure conformity to the provisions of this chapter. There should be a process for the identification of resources needed for adherence to GMP. A gap analysis based on audits by internal personnel, customers, regulatory agencies, or outside contractors, or based on the use of this chapter, could be created to identify resource requirements. Top management should ensure that the integrity of the quality management system is maintained when changes are planned and implemented.

Responsibility, Authority, and Communication

RESPONSIBILITY AND AUTHORITY

Responsibility and authority should be clearly defined by top management and communicated within the organization. It should be the responsibility of a unit that is independent of production, such as the quality unit, to do the following:

- ensure that quality-critical activities are undertaken as defined,
- approve suppliers of quality-critical materials and services,
- approve or reject raw materials, packaging components, intermediates, and finished excipients,
- ensure that there is a review of production records to confirm that no errors have occurred or, if errors have occurred, that they are fully investigated,
- participate in reviewing and authorizing changes to processes, specifications, procedures, and test methods that potentially affect quality (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) and participate also in investigating failures and complaints,
- retain responsibility for approval or rejection of the excipient if it is produced, processed, packaged, or held under contract by another company,
- develop and implement a self-inspection program of the quality management system.

The excipient manufacturer may delegate some of the quality unit's activities to other personnel if appropriate controls (for example, periodic audits, training, and documentation) are in place.

An organization chart by function should show interdepartmental relationships as well as relationships to top management of the company. Personnel whose positions affect excipient quality should have job descriptions.

MANAGEMENT REPRESENTATIVE

The excipient manufacturer should appoint a management representative with sufficient authority to ensure that the provisions of this chapter are properly implemented. The representative should periodically report to top management on conformity to the quality management system, including changing customer and regulatory requirements.

INTERNAL COMMUNICATION

The excipient manufacturer should ensure that appropriate systems are established to communicate GMP and regulatory requirements, quality policies, quality objectives, and procedures throughout the organization. The communication should also provide information about the effectiveness of the quality management system. Top management should be notified promptly of quality-critical situations, such as product retrievals, in accordance with a documented procedure.

Management Review

GENERAL

The top management of the company should hold periodic reviews of the quality management system to confirm the organization's continued conformity to this chapter. The review should be recorded and should include assessing opportunities for improvement and the need for changes to the quality management system.

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REVIEW INPUT

Management review inputs should include, for example, the following:

- results of internal and external audits,
- customer feedback of the company performance,
- product conformity and process performance,
- action items from the previous management review,
- customer complaints,
- status of corrective or preventive actions,
- changes that could affect the quality management system.

REVIEW OUTPUT

The management review should identify the resources needed and the opportunities presented for improving the quality management system and improving product conformity to customer and regulatory requirements. A record should be made of actions recommended and taken.

RESOURCE MANAGEMENT

Provision of Resources

There should be sufficient qualified personnel and resources (e.g., equipment, materials, buildings, and facilities) to implement, maintain, and improve the quality management system and to produce, package, test, store, and release each excipient in a manner consistent with this chapter.

Human Resources

GENERAL

Personnel performing work affecting the quality of excipients should have the appropriate combination of education, training, and experience for their assigned tasks. Consultants advising on the design, production, packaging, testing, or storage of excipients should have sufficient education, training, and experience or any combination thereof to advise on the subject for which they are retained. Records should be maintained listing the name, address, and qualifications of consultants and the type of service they provide.

COMPETENCE, AWARENESS, AND TRAINING

The excipient manufacturer should establish and maintain procedures for identifying training needs and for providing the necessary training to personnel performing activities affecting excipient quality. Appropriate records of training should be maintained. Training should address the particular operations that the employee performs and GMP as they relate to the employee's functions. Qualified individuals should conduct GMP training frequently enough to ensure that employees remain familiar with applicable GMP principles. Management should establish adequate and continued personal-hygiene training for personnel who handle materials so that they understand the precautions necessary for preventing contamination of excipients. The training program should ensure that personnel understand that deviations from procedures may affect the customer's product quality.

PERSONNEL HYGIENE

To protect excipients from contamination, protective apparel such as head, face, hand, and arm coverings should be worn as appropriate to the duties performed. Jewelry and other loose items, including those in pockets, should be removed or covered. Only authorized personnel should enter the areas of the buildings and facilities designated as limited-access areas.

Personnel should practice good sanitation and health habits. Any person shown by either medical examination or supervisory observation to have an apparent illness or open lesions that may adversely affect the safety or quality of the excipient should be excluded from direct contact with raw materials, packaging components, intermediates, and finished excipients until the condition is corrected or until competent personnel determine that it will not jeopardize the safety or quality of the excipient. Personnel should be instructed to report to supervisory personnel any health conditions that may have an adverse effect on excipients. The storage and use of food, drink, personal medication, tobacco products, or similar items should be restricted to designated locations separate from manufacturing areas.

INFRASTRUCTURE

The infrastructure should be managed, operated, cleaned, and maintained in accordance with GMP principles to ensure excipient quality and to avoid contamination (including, where critical to excipient quality, control of particulate matter, microbiological control, and control of water quality).

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BUILDINGS AND FACILITIES

The prevention of contamination should be considered in the design of the manufacturing processes and facilities, particularly when the excipient is exposed. Buildings and facilities used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation appropriate to the type of processing.

Manufacturing processes associated with the production of highly sensitizing or toxic products (for example, herbicides and pesticides) should be located in dedicated facilities or should use equipment separate from that used for excipient manufacture. If this is not possible, appropriate measures (for example, cleaning, inactivation) should be implemented to avoid cross-contamination. The effectiveness of these measures should be demonstrated. There should be adequate facilities for the testing of raw materials, packaging components, intermediates, and finished excipients.

EQUIPMENT

Equipment used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation, depending on the type of processing (for example, batch versus continuous). Equipment should be commissioned before use to ensure that it is functioning as intended. Where equipment is located outdoors, there should be suitable controls to minimize the risk to excipient quality from the environment (for example, processing within a closed system).

EQUIPMENT CONSTRUCTION

Process equipment should be constructed so that contact surfaces will not be reactive, additive, or absorptive and thus will not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, should preferably not come into contact with raw materials, packaging materials, intermediates, or finished excipients. Where contact is possible, substances suitable for use in food applications should be employed.

Equipment should be designed to minimize the possibility of contamination caused by direct operator contact in activities such as the unloading of centrifuge bags, the use of transfer hoses (particularly those used to transfer powders), and the operation of drying equipment and pumps. The sanitary design of transfer and processing equipment should be evaluated. To control the risk of contamination, equipment with moving parts should be assessed with regard to the integrity of seals and packing materials.

EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the production, processing, packaging, testing, or holding of the excipient. There should be records of the use and maintenance of quality-critical equipment. These records can be in the form of a log, computer database, or other appropriate documentation.

COMPUTER SYSTEMS

Computer systems that may affect excipient quality should have sufficient controls for operation and maintenance and for prevention of unauthorized access or changes to computer software, hardware, or data, including the following:

- systems and procedures that show that the equipment and software are performing as intended,
- procedures for checking the equipment at appropriate intervals,
- retention of suitable back-up or archival systems such as copies of the program and files,
- assurance that changes are verified and documented and are made only by authorized personnel.

UTILITIES

Utilities (for example, nitrogen, compressed air, and steam) used in the production, storage, or transfer of materials that could affect excipient quality should be assessed and appropriate action taken to control the risk of contamination and cross-contamination.

WATER

Water used in the manufacture of excipients should be demonstrated to be of appropriate quality in consideration of purity requirements and the intended use of the excipient. Unless otherwise justified, process water should, at a minimum, meet regulatory requirements for drinking (potable) water. If drinking (potable) water is insufficient to ensure quality, or if tighter chemical and/or microbiological water quality specifications are required, appropriate controls and specifications should be set: for example, physical and chemical attributes, total microbial counts, and limits on objectionable organisms and/or endotoxins.

Where water used in the process is treated by the manufacturer to achieve a defined quality, the treatment process should be specified and monitored with appropriate action limits. Water that comes into contact with the excipient should be supplied under continuous positive pressure (or other means of preventing back flow) in a system free of defects to control the risk of contamination to the excipient.

WORK ENVIRONMENT

Where the excipient is exposed during manufacture, it should be in an environment appropriate for minimizing contamination. The manufacturer should apply suitable controls to maintain that environment.

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AIR HANDLING

Where an air-handling system is installed to provide protection to the excipient, the excipient manufacturer should demonstrate its effectiveness. Excipient production unit air-handling systems should be designed to prevent cross-contamination. For dedicated areas processing the same excipient, it is permissible to recycle a portion of the exhaust air back into the same area. The adequacy of such a system for multiuse areas, especially if several products are processed simultaneously, should be assessed for potential cross-contamination.

CONTROLLED ENVIRONMENT

A controlled environment may be necessary in order to avoid contamination or degradation caused by exposure to heat, air, or light. The degree of protection required may vary depending on the stage of the process. Special environments required by some processes should be monitored to ensure product quality (for example, inert atmosphere or protection from light). Where an inert atmosphere is required, the gas should be treated as a raw material. If interruptions in the special environment occur, adequate evidence and an appropriate rationale should be documented to show that such interruptions have not compromised the quality of the excipient. Such environmental concerns become increasingly important following purification of the excipient.

CLEANING AND SANITARY CONDITIONS

Adequate cleanliness is an important consideration in the design of excipient manufacturing facilities. Buildings used in the production, processing, packaging, or holding of an excipient should be maintained in an appropriately clean and sanitary condition according to the type of processing conducted (for example, open/closed systems). Where maintenance of clean and sanitary conditions is critical to excipient quality, documented procedures should assign responsibility for cleaning and sanitation, describing in sufficient detail the cleaning schedules, methods, equipment, and materials to be used in cleaning the buildings and facilities. These procedures should be followed, and cleaning should be documented. Waste should be segregated and disposed of in a timely and appropriate manner. If waste is not disposed of immediately, it should be suitably identified.

PEST CONTROL

Buildings should be free from infestation by rodents, birds, insects, and other vermin. Some raw materials, particularly botanicals, may contain some unavoidable contamination, such as rodent or other animal filth or infestation. The manufacturer should have sufficient control methods to prevent the increase of such contamination or infestation in holding areas and its spread to other areas of the plant.

LIGHTING

Adequate lighting should be provided to facilitate cleaning, maintenance, and proper operations.

DRAINAGE

In areas where the excipient is open to the environment, drains should be of adequate size and, where connected directly to a sewer, should be provided with an air break or other mechanical device to prevent back-siphoning.

WASHING AND TOILET FACILITIES

Adequate personal washing facilities should be provided, including hot and cold water, soap or detergent, air dryers or single-service towels, and clean toilet facilities easily accessible to working areas. Adequate facilities for showering and/or changing clothes should be provided, where appropriate.

PRODUCT REALIZATION

Planning of Product Realization

The excipient manufacturer should plan and develop the processes and controls needed for product manufacture. These plans and controls should be appropriate to the production process, excipient specification, equipment, and facilities used in the manufacture of the product. Key aspects of the planning of a suitable process and its controls should include the following, as appropriate:

- documented testing programs, for quality-critical materials including excipients, that include appropriate specifications, sampling plans, and test and release procedures,
- generation and maintenance of records (also see above, *Control of Records* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) that provide evidence that these plans have been realized as intended and that enable traceability to be demonstrated (also see below in this section, *Traceability* under *Identification and Traceability*),
- provision of resources to implement these plans,
- environmental and hygiene control programs to minimize contamination.

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Customer-Related Processes

DETERMINATION OF REQUIREMENTS RELATED TO THE PRODUCT

The excipient manufacturer should determine the excipient quality, labeling, and delivery requirements of the customer. Additional requirements, whether customer-specific, legal, or regulatory (for example, pharmacopeia material and general monographs), should be agreed on by both parties. Requirements not stated by the customer but necessary for specified or intended use, where known, should be considered.

REVIEW OF REQUIREMENTS RELATED TO THE PRODUCT

The excipient manufacturer and customer should mutually agree upon the requirements identified in the section above, *Determination of Requirements Related to the Product*, before supply commences. The manufacturer should have the facility and process capability to consistently meet the mutually agreed-upon specifications. Where the requirements determined in the section *Determination of Requirements Related to the Product* are changed, this review should be repeated before supply recommences.

CUSTOMER COMMUNICATION

There should be provision for providing accurate and pertinent communication to the customer. Master copies of documents such as specifications and technical reports should be controlled documents. Provision should be made for replying to customer inquiries, contracts, and order-handling requirements. Customer feedback and complaints should be documented. Customers should be notified of significant changes (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*).

DESIGN AND DEVELOPMENT

ISO 9001 includes requirements for ensuring control over design and development activities. It is recommended that companies involved in such activities follow the requirements of ISO 9001. Full GMP are not always applicable during the design and development of new excipients and/or manufacturing processes. However, development batches of excipients that are intended for use in drug products should be manufactured in accordance with the applicable provisions of this chapter.

Purchasing

PURCHASING PROCESS

Excipient manufacturers should have a system for selecting and approving suppliers of quality-critical materials and services (for example, subcontract manufacturers and laboratories). Supplier approval by the quality unit should require an evaluation of the supplier's quality management system, including adequate evidence that they can consistently meet agreed-upon specifications and maintain traceability. This may require periodic audits of the supplier's manufacturing facility. Records of these activities should be maintained. Materials should be purchased against an agreed specification from approved suppliers.

PURCHASING INFORMATION

Purchasing agreements should describe the material or service ordered, including, where critical to excipient quality, the following:

- the name, type, class, style, grade, item code number or other precise identification traceable to the raw material and packaging specifications,
- drawings, process requirements, inspection instructions and other relevant technical data, including requirements for approval or qualification of product, procedures, process equipment, and personnel,
- adherence to the appropriate sections of this chapter for relevant contract manufacturers or laboratories, and
- a statement to notify the excipient manufacturer of significant changes in quality-critical raw materials.

VERIFICATION OF PURCHASED PRODUCT

There should be procedures for the approval and release of quality-critical material. Upon receipt, quality-critical materials should be placed in quarantine and should not be used prior to acceptance. Effective quarantine can be established with suitable identifying labels, signs, and/or other manual documentation systems. When quarantine and stock control are managed with computer systems in lieu of a physical stock control, system controls should prevent the use of unreleased material. Quarantine may not be feasible for materials supplied via pipelines. In these cases the excipient manufacturer should establish an agreement with the supplier so that the manufacturer is notified of material that does not meet specification. Sampling activities should be conducted under defined conditions, in accordance with a defined sampling method and using procedures designed to prevent contamination and cross-contamination.

Quality-critical materials used in the manufacture of an excipient should be tested or otherwise verified prior to use. Verification should include availability and a check of the supplier certificate of analysis and, wherever feasible, at least an identification test. Testing schedules should be organized to separate routine tests from those that are performed infrequently or only for new suppliers. Bulk deliveries should have additional controls to ensure material purity and freedom from

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contamination (for example, dedicated tankers, tamper-evident seals, a certificate of cleaning, analytical testing, or audit of the supplier). These procedures, activities, and results should be documented.

Production and Service Provision

CONTROL OF PRODUCTION AND SERVICE PROVISION

Production activities should be carried out under controlled conditions (also see above, *Planning of Product Realization* under *Product Realization*). Specific examples of important controls, some of which may not be applicable to all excipient manufacturers, are illustrated in the following sections.

PRODUCTION INSTRUCTIONS AND RECORDS

Production instructions and records are required but may differ for the type of operation: for example, batch versus continuous processes. There should be a controlled document that describes how the excipient is produced (for example, master production instructions, master production and control records, or process definitions). For batch processes, an accurate reproduction of the appropriate master production instructions should be issued to the production area. For continuous processes, a current processing log should be available. Records should be available for each batch of excipient produced and should include complete information relating to the production and control of each batch. For continuous processes, the batch and its records should be defined (for example, based on time or defined quantity). Records may be in different locations but should be readily retrievable. Records for both batch and continuous processing, where critical to excipient quality, should include the following:

- date and time each step was completed or date and time log of key parameters,
- identification of persons performing and directly supervising or checking each significant step, operation or control parameter,
- identification of major equipment and lines used,
- material inputs to enable traceability: for example, batch number and quantities of raw material/intermediate and time it was added,
- in-process and laboratory control results,
- the quantity produced for the defined batch and a statement of the percentage of theoretical yield, unless not quantifiable (for example, as in some continuous processes),
- inspection of the packaging and labeling area before and after use,
- labeling control records,
- description of excipient product containers and closures,
- description of sampling performed,
- failures, deviations and their investigations,
- results of final product inspection.

EQUIPMENT CLEANING

The manufacturer should design and justify cleaning and sanitization procedures and provide evidence of their effectiveness. In multipurpose plants the use of the *model product* approach (groups of product of similar type) may be used in justifying a suitable procedure. Cleaning and sanitization procedures should be documented. They should contain sufficient detail to allow operators to clean each type of equipment in a reproducible and effective manner. There should be a record confirming that these procedures have been followed. Equipment and utensils should be cleaned and sanitized where critical to excipient quality and at appropriate intervals to prevent contamination and cross-contamination of the excipient. The cleaning status of equipment should be recorded appropriately.

Where multipurpose equipment is in use, it is important to be able to determine previous usage when investigating cross-contamination or the possibility of such contamination (also see below in this section, *Records of Equipment Use*). During a production campaign, incidental carryover frequently occurs, and it is usually acceptable because cleanup between successive batches of the same excipient is not normally required in order to maintain quality levels. Products that leave residues that cannot be effectively removed should be produced in dedicated equipment. For continuous processing, the frequency of equipment cleaning should be determined by the manufacturer and justified.

RECOVERY OF SOLVENTS, MOTHER LIQUORS, AND SECOND CROP CRYSTALLIZATIONS

Where solvents are recovered and reused in the same process or different processes, they should meet appropriate standards prior to reuse or mixing with other approved material. Mother liquors or filtrates containing recoverable amounts of excipient, reactants, or intermediates are frequently reused. Such processes should be documented in the production records or logs to enable traceability.

IN-PROCESS BLENDING OR MIXING

In-process blending or mixing to ensure batch uniformity or to facilitate processing should be controlled and documented. If the intent of the operation is to ensure batch uniformity, it should be performed so as to ensure homogeneous mixing of materials to the extent feasible and should be reproducible from batch to batch.

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IN-PROCESS CONTROL

In-process inspection and testing, based on monitoring the process or actual sample analysis at defined locations and times, should be performed. Sampling methods should be documented to ensure that the sample is representative and clearly labeled. In-process samples should not be returned to production for incorporation into the final batch.

The results of in-process tests should be recorded and should conform to established process parameters or acceptable tolerances. Work instructions should define the procedure to follow and should indicate how to use the inspection and test data to control the process. There should be defined actions to be taken when the results are outside specified limits. Where approval to continue with the process is issued within the production department, the specified tests should be performed by trained personnel and the results recorded.

PACKAGING AND LABELING

Procedures should be employed to protect the quality and purity of the excipient when it is packaged and to ensure that the correct label is applied to all containers. Packaging and labeling operations should be designed to prevent mix-ups. Procedures should be implemented to ensure that the correct labels are printed and issued and that the labels contain the correct information. The procedure should also specify that excess labels are immediately destroyed or returned to controlled storage. Excess labels bearing batch numbers should be destroyed. Packaging and labeling facilities should be inspected immediately before use to ensure that materials that are not required for the next packaging operation have been removed. When excipients are labeled on the packaging line, packaged in preprinted bags, or bulk-shipped in tank cars, there should be documentation of the system used to satisfy the intent of the above procedures.

RECORDS OF EQUIPMENT USE

Records of quality-critical equipment use should be retained. These records should allow the sequence of cleaning, maintenance, and production activities to be determined.

VALIDATION OF PROCESSES FOR PRODUCTION AND SERVICE PROVISION

An important factor in the assurance of product quality includes the adequate design and control of the manufacturing process, because product testing alone is not sufficient to reveal variations that may have occurred. Each step of the manufacturing process should be controlled to the extent necessary for ensuring that the excipient meets established specifications. The concept of process validation is a key element in ensuring that these quality assurance goals are met. The process reactions, operating parameters, purification steps, impurities, and key tests needed for process control should be documented, thus providing the basis for validation.

The full validation program that is typically performed in the pharmaceutical industry may not always be carried out by the excipient manufacturer. However, the excipient manufacturer should demonstrate the consistent operation of each manufacturing process: for example, through process capability studies, development, and scale-up reports.

Identification and Traceability

TRACEABILITY

Quality-critical items (for example, raw materials, packaging materials, intermediates, and finished excipients) should be clearly identified and traceable through records. These records should allow traceability of the excipient both upstream and downstream. Identification of raw materials used in batch production processes should be traceable through the batch numbering system or other appropriate system. Identification of raw materials used in excipients produced by continuous processing should indicate the time frame during which a particular batch of raw material was processed through the plant. Excipient manufacturers should also have adequate knowledge about the origin of any raw materials derived from plant or animal matter.

Raw materials, including solvents, are sometimes stored in bulk tanks or other large containers, making precise separation of batches difficult. Nevertheless, the use of such materials and containers should be documented in production records.

INSPECTION AND TEST STATUS

There should be a system for identifying the inspection status of quality-critical items, including raw materials, packaging materials, intermediates, and finished excipients. Although storing materials in identified locations is preferred, any means that clearly identifies the test status is satisfactory. Continuously fed materials may need special consideration in order to satisfy these requirements.

LABELING

Labeling for excipient packages is subject to national and international regulatory requirements, which may include transportation and safety measures. At a minimum, labels should include the following:

- the name of the excipient and grade, if applicable,
- the excipient manufacturer's and/or distributor's name,
- the batch number from which the complete batch history can be determined,
- special storage conditions, if applicable.

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CUSTOMER PROPERTY

The excipient manufacturer should establish and maintain procedures for verification, storage, and maintenance of customer-supplied materials intended for incorporation into the customer's excipient. Verification by the manufacturer does not relieve the customer of the responsibility of providing an acceptable material. Material that is lost or that is damaged or otherwise unsuitable for use should be recorded and reported to the customer. In this case, procedures should be in place for acceptable disposition and replacement of the material. The manufacturer should also make provisions for protecting other real and intellectual property that is provided by the customer (for example, test equipment, test methods, and specifications).

Preservation of Product

HANDLING, STORAGE, AND PRESERVATION

Excipients, intermediates, and raw materials should be handled and stored under appropriate conditions of temperature, humidity, and light so that their identity, quality, and purity are not affected. Outdoor storage of raw materials (for example, acids, other corrosive substances, explosive materials) or excipients is acceptable, provided that the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, and containers are adequately cleaned prior to opening and use. Records of storage conditions should be maintained if they are critical for the continuing conformity of the material to specifications.

PACKAGING SYSTEMS

An excipient packaging system should include the following features:

- documented specifications and examination or testing methods,
- cleaning procedures, where containers are reused,
- tamper-evident seals,
- containers that provide adequate protection against deterioration or contamination of the excipient during transportation and recommended storage,
- containers that do not interact with or contaminate the excipient,
- storage and handling procedures that protect containers and closures and minimize the risk of contamination, damage, or deterioration and that will avoid mix-ups (for example, between containers that have different specifications but are similar in appearance).

If returnable excipient containers are reused, previous labeling should be removed or defaced. If the containers are reused solely for the same excipient, previous batch numbers or the entire label should be removed or completely obliterated.

DELIVERY AND DISTRIBUTION

Identification and traceability of quality-critical aspects are required of excipient manufacturers. Distribution records of excipient shipments should be kept. These records should identify, by excipient batch, where and to whom the excipient was shipped, the amount shipped, and the date of shipment so as to facilitate retrieval if necessary. Where excipients are handled by a series of different distributors, it should be possible to trace them back to the original manufacturer, and not only to the previous supplier. The manufacturer should maintain the integrity and the quality of the product after final inspection and test. Where contractually specified, this protection should be extended to include delivery to the final destination. Excipients should be supplied only within their expiry and/or retest period.

CONTROL OF MEASURING AND MONITORING DEVICES

Measuring and test equipment, including computerized systems, identified as being quality-critical should be calibrated and maintained. This includes in-process instruments as well as test equipment used in the laboratory. The control program should include the standardization or calibration of instruments and equipment at suitable intervals in accordance with an established, documented program. This program should contain specific directions, schedules, limits for accuracy and precision, and provisions for remedial action in the event that accuracy and/or precision limits are not met. Calibration standards should be traceable to recognized national or compendial standards as appropriate.

Instruments and equipment not meeting established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration status of quality-critical equipment should be known and verifiable to users.

MEASUREMENT, ANALYSIS, AND IMPROVEMENT

The organization should plan and implement the monitoring, measurement, and improvement activities that are required in order to demonstrate conformity of the excipient to customer requirements and to ensure conformity of the quality management system to this chapter. The organization should evaluate opportunities for improvements through the measurement and analysis of product and process trends.

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Monitoring and Measurement

CUSTOMER SATISFACTION

The excipient manufacturer should establish measurement activities to assess customer satisfaction. Such measurements can include customer complaints, return of excipients, and customer feedback. This information should drive activities that strive to continuously improve customer satisfaction.

INTERNAL AUDIT

The excipient manufacturer should carry out a comprehensive system of planned and documented internal quality audits. These should determine whether quality activities comply with planned arrangements and should also determine the effectiveness of the quality management system. Audits should be scheduled on the basis of the status and importance of the activity. Audits and follow-up actions should be carried out in accordance with documented procedures. Audit results should be documented and discussed with management personnel having responsibility in the area audited. Management personnel responsible for the area audited should take corrective action on the nonconformities found. *Appendix: Auditing Considerations* will be of assistance in establishing an internal audit program.

MONITORING AND MEASUREMENT OF PROCESSES

The excipient manufacturer should identify the tests and measurements necessary for adequately controlling manufacturing and quality management system processes. When critical to excipient quality, techniques used to verify that the processes are under control should be established. When deviations from planned results occur, corrective action should be taken to ensure that the excipient meets requirements. Periodic reviews of key indicators such as process quality attributes and process failures should be conducted to assess the need for improvements.

MONITORING AND MEASUREMENT OF PRODUCT

The excipient manufacturer should establish the test methods and procedures to ensure that the product consistently meets specifications. Analytical methods should be suited to their purposes. The analytical methods may be those included in the current edition of the appropriate pharmacopeia or another accepted standard. However, the methods may also be noncompendial. If the excipient manufacturer claims that its product is in compliance with a pharmacopeia or an official compendium, then

- noncompendial analytical tests should be demonstrated to be equivalent to those in the compendia;
- the product should comply with applicable *USP* general chapters and notices.

LABORATORY CONTROLS

Laboratory controls should include complete data derived from tests necessary for ensuring conformity with specifications and standards, including the following:

- a description of the sample received for testing, together with the material name, a batch number or other distinctive code, and the date the sample was taken,
- a statement referencing each test method used,
- a record of raw data secured during each test, including graphs, chromatograms, charts, and spectra from laboratory instrumentation, identified to show the specific material and batch tested,
- a record of calculations performed in connection with the test,
- test results and how they compare with established specifications,
- a record of the person who performed each test and the date(s) the tests were performed.

There should be a documented procedure for the preparation of laboratory reagents and solutions. Purchased reagents and solutions should be labeled with the proper name, concentration, and expiry date. Records should be maintained for the preparation of solutions and should include the name of the solution, the date of preparation, and the quantities of material used. Volumetric solutions should be standardized according to an internal method or by using a recognized standard. Records of the standardization should be maintained.

Where used, primary reference reagents and standards should be appropriately stored and need not be tested upon receipt, provided that a certificate of analysis from the supplier is available. Secondary reference standards should be appropriately prepared, identified, tested, approved, and stored. There should be a documented procedure for the qualification of secondary reference standards against primary reference standards. The reevaluation period should be defined for secondary reference standards, and each batch should be periodically requalified in accordance with a documented protocol or procedure.

FINISHED EXCIPIENT TESTING AND RELEASE

Finished excipient testing should be performed on each batch to ensure that the excipient conforms to documented specifications. There should be a procedure to ensure that appropriate manufacturing documentation, in addition to the test results, is evaluated prior to release of the finished excipient. The quality unit should be responsible for the release of the finished excipient. For excipients produced by continuous processes, assurance that the excipient conforms to documented specifications may be achieved through the results of in-process testing or other process control records.

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OUT-OF-SPECIFICATION TEST RESULTS

Out-of-specification (OOS) test results should be investigated and documented according to a documented procedure. Retest sample results may be used to replace the original test result only if it is demonstrated on the basis of a documented investigation that the original result is erroneous. When statistical analysis is used, both the original and retest data must be included. The OOS procedure should define which statistical techniques are to be used and under what circumstances. These same principles apply when the sample is suspected of not being representative of the material from which it was taken.

RETAINED SAMPLES

When practical, a representative sample of each batch of the excipient should be retained. The retention period should be appropriate to the expiry or reevaluation date. The retained samples should be stored and maintained in such a manner that they are readily retrievable in facilities that provide a suitable environment. The sample size should be at least twice the amount required to perform complete specification testing.

CERTIFICATES OF ANALYSIS

The organization should provide certificates of analysis to the required specification for each batch of excipient.

IMPURITIES

When possible, excipient manufacturers should identify and set appropriate limits for impurities. The limits should be based on appropriate safety data, limits as described in official compendia or other requirements, and sound GMP considerations. Manufacturing processes should be adequately controlled so that the impurities do not exceed such established limits. Many excipients are extracted from or purified using organic solvents. These solvents are normally removed by drying. It is important that excipient specifications include tests and limits for solvent residues.

STABILITY

Although many excipient products are stable and may not require extensive testing to ensure stability, the stability of excipients is an important factor in the overall quality of the drug product. For excipients that have been on the market for a long time, historical data may be used to indicate stability. Where historical data do not exist, a documented testing and/or evaluation program designed to assess the stability characteristics of the excipient should be undertaken. The results of such stability testing and/or evaluation should be used in determining appropriate storage conditions and retest or expiry dates. The testing program should include the following:

- the number of batches, sample sizes and test intervals,
- storage conditions for samples retained for testing,
- suitable stability-indicating test methods,
- storage of the excipient in containers that simulate the market container, where possible.

The stability of excipients may be affected by undetected changes in raw materials or subtle changes in manufacturing procedures or storage conditions. Excipients may also be shipped in a variety of packaging types that can affect their stability (for example, plastic or glass bottles, metal or plastic drums, bags, tank cars, or other bulk containers).

Some excipients may be available in different grades (for example, various molecular weights of a polymer or different monomer ratios, different particle sizes, bulk densities) or may be mixtures of other excipients. These excipients may be very similar to others within a product group. Minor quantitative differences of some of the components may be the only significant variation from one product to another. For these types of excipients, a model product approach may be appropriate for assessment of the stability of similar excipients. Stability studies of this type should involve selection of several model products that would be expected to simulate the stability of the product group being assessed. This selection should be scientifically sound and documented. Data from stability studies of these model products can be used to determine theoretical stability for similar products.

EXPIRY/RETEST PERIODS

An expiry or retest period should be assigned to each excipient and communicated to the customer. Common practice is to use a retest period rather than an expiry period.

CONTROL OF NONCONFORMING PRODUCT

Raw material, intermediate, or finished excipient found not to meet its specifications should be clearly identified and controlled to prevent inadvertent use or release for sale. A record of nonconforming product should be maintained. Incidences of nonconformity should be investigated to identify the cause. The investigation should be documented and action taken to prevent recurrence. There should be a documented procedure defining how the retrieval of an excipient from distribution should be conducted and recorded. Procedures should exist for the evaluation and subsequent disposition of nonconforming products. Nonconforming product should be reviewed in accordance with documented procedures to determine if it can be

- reprocessed or reworked to meet the specified requirements,
- accepted by the customer with customer agreement,
- regraded for other applications,
- destroyed.

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REPROCESSING

Repetition of an activity that is a normal part of the manufacturing process (reprocessing) should occur only when it has already been documented that the excipient may be made in that manner. In all other cases, the guidance for reworking should be followed.

REWORKING

An activity that is not a normal part of the manufacturing process (reworking) should be conducted only following a documented review of risk to excipient quality and approval by the quality unit. As appropriate, when performing the risk assessment, consideration should be given to the following:

- new impurities that may be introduced as a result of reworking,
- additional testing to control the reworking,
- records and traceability to the original batches,
- suitable acceptance criteria for the reworked excipient,
- impact on stability or the validity of the reevaluation interval,
- performance of the excipient.

When the need to rework an excipient is identified, an investigation and evaluation of the cause are required. The equivalence of the quality of reworked material to original material should also be evaluated and documented to ensure that the batch will conform to established specifications and characteristics. Batches of excipients that do not conform to specifications individually must not be blended with other batches that do conform in an attempt to hide adulterated or substandard material.

RETURNED EXCIPIENTS

Returned excipients should be identified and quarantined until the quality unit has completed an evaluation of their quality. There should be procedures for holding, testing, reprocessing, and reworking of the returned excipient. Records for returned products should be maintained and should include the name and the batch number of the excipient, the reason for the return, the quantity returned, and the ultimate disposition of the returned excipient.

ANALYSIS OF DATA

The excipient manufacturer should develop methods for evaluating the effectiveness of its quality management system and use those data to identify opportunities for improvement. Such data can be derived from customer complaints, product reviews, process capability studies, internal audits, and customer audits. The analysis of such data may be used as part of the management review (also see above, *Management Review* under the *Management Responsibility* section). A periodic review of key indicators such as product quality attributes, customer complaints, and product nonconformities may be conducted to assess the need for improvements.

Improvement

CONTINUAL IMPROVEMENT

The excipient manufacturer should take proactive measures to continuously improve manufacturing and quality management system processes. To identify opportunities for continual improvement, analysis of the following performance indicators may be considered:

- causes of nonconforming product,
- results of internal and external audits,
- customer returns and complaints,
- process and operational failures.

Corrective Action: The excipient manufacturer should establish, document, and maintain procedures for the following:

- determining the root causes of nonconformities,
- ensuring that corrective actions are implemented and effective,
- implementing and recording changes in procedures resulting from corrective action.

Preventive Action: The excipient manufacturer should establish, document, and maintain procedures for the following:

- initiating preventive actions to deal with problems at a level corresponding to the risks,
- implementing and recording changes in procedures resulting from preventive action.

GLOSSARY

The terms below are defined as used in this chapter. Wherever possible, definitions used by the International Conference on Harmonization have been used as the basis for the glossary.

Acceptance Criteria: numerical limits, ranges, or other suitable measures of acceptance for test results.

Active Pharmaceutical Ingredient (API): any substance or mixture of substances that is intended to be used in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

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Adulterated Material: a material that either has been contaminated with a foreign material or has not been manufactured using GMP. This does not pertain to a material that simply does not meet physical or chemical specifications.

Batch (Lot): a specific quantity of material produced in a process or series of processes so that it can be expected to be homogeneous. In the case of continuous processes, a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval.

Batch Number (Lot Number): a unique combination of numbers, letters, and/or symbols that identifies a batch and from which the production and distribution history can be determined.

Batch Process: a process that produces the excipient from a discrete supply of raw materials that are present before the completion of the reaction.

Batch Record: documentation that provides a history of the manufacture of a batch of excipient.

Blending (Mixing): intermingling different conforming grades into a homogeneous lot.

Calibration: the demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard, over an appropriate range of measurements.

CEP (Certificate of Suitability to the *European Pharmacopoeia*): certification granted to individual manufacturers by the European Directorate for the Quality of Medicines (EDQM) when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

Certificate of Analysis: a document listing the test methods, specification, and results of testing a representative sample from the batch to be delivered.

Commissioning: the introduction of equipment for use in a controlled manner.

Contamination: the undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

Continuous Process: a process that continuously produces material from a continuing supply of raw material.

Critical: a process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

Cross-Contamination: contamination of a material or product with another material or product.

Customer: the organization receiving the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

Deviation: departure from an approved instruction or established standard.

Drug Master File (DMF): detailed information about the manufacture of an excipient that is submitted to the U.S. Food and Drug Administration (FDA).

Drug (Medicinal) Product: the dosage form in the final immediate packaging intended for marketing.

Excipient: substances other than the API that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.

Expiry (Expiration) Date: the date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

Impurity: a component of an excipient that is not intended to be present but arises as a consequence of the manufacturing process.

In-Process Control/Testing: checks performed during production to monitor and, if appropriate, to adjust the process and/or to ensure that the intermediate or excipient conforms to its specification.

Intermediate: material that must undergo further manufacturing steps before it becomes an excipient.

Lot: See *Batch*.

Manufacturer/Manufacturing Process: all operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.

Master Production Instruction (Master Production and Control Record): documentation that describes the manufacture of the excipient from raw material to completion.

Material: a general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.

Model Product: a product that represents a group of similar products with respect to composition, functionality, or specification.

Mother Liquor: the residual liquid that remains after crystallization or isolation processes.

Packaging Material: a material intended to protect an intermediate or excipient during storage and transport.

Production: operations involved in the preparation of an excipient from receipt of materials through processing and packaging of the excipient.

Quality Assurance: the sum total of the organized arrangements made with the object of ensuring that all excipients are of the quality required for their intended use and that quality systems are maintained.

Quality Control: checking or testing that specifications are met.

Quality-Critical: describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.

Quarantine: the status of materials isolated physically or by other effective means pending a decision on their subsequent approval or rejection.

Raw Material: a general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.

Record: a document stating results achieved and/or providing evidence of activities performed. The medium may be paper, magnetic, electronic or optical, photographic, or another medium, or a combination thereof.

Reevaluation Date (Retest Date): the date when the material should be reexamined to ensure that it is still in conformity with the specification.

Reprocessing: repetition of an activity that is a normal part of the manufacturing process and that has been documented previously.

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Retrieval: process for the removal of an excipient from the distribution chain.

Reworking: subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.

Specifications: list of tests, references to analytical procedures, and appropriate acceptance criteria that are numerical limits, ranges, or other criteria for the tests described for a material.

Stability: continued conformity of the excipient to its specifications.

Top Management: person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and will depend on the way in which the quality management system is organized.

Traceability: ability to determine the history, application, or location that is under consideration: for example, origin of materials and parts, processing history, or distribution of the product after delivery.

Validation: a documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

APPENDIX

Auditing Considerations

INTRODUCTION

Many excipients are used in food, cosmetic, and industrial products as well as in pharmaceuticals. Thus, environmental conditions, equipment, and operational techniques employed in excipient manufacture are often those of the chemical industry as opposed to the pharmaceutical industry. Chemical processes can produce impurities from side reactions. Careful process control is therefore essential to minimize levels of impurities and contamination.

Excipients are often manufactured on a large scale, using continuous processing and automated process controls. Production equipment and processes vary depending on the type of excipient being produced, the scale of production, and the type of operation (for example, batch versus continuous process).

This appendix is intended as an aid in preparing for an audit of an excipient manufacturer. Both external and internal auditors (see also *Internal Audit* in *Monitoring and Measurement* under the *Measurement, Analysis, and Improvement* section) will find this appendix useful in identifying the significant issues with respect to GMP and quality that require examination. This section will assist excipient manufacturers in identifying key deliverables when adopting the GMP standards listed in the other sections of this chapter; in planning an audit, it will also help to verify the quality of the excipient manufacturing process and the manufacturer's quality management system.

GMP PRINCIPLES

Control of Impurities and Contamination: In general, the pharmaceutical customer does not perform further chemistry or purification steps on the excipient; it is used as purchased. Consequently, impurities present in the excipient are likely to be present in the drug product. Although dosage form manufacturers have some control over excipient quality through specifications, excipient manufacturers have greater control over the physical characteristics, quality, and presence of impurities in the excipients they produce.

External contamination of the excipient can arise from the manufacturing environment. However, chemical processes used to manufacture excipients are often performed in closed systems that afford protection against such contamination, even when the reaction vessels are not located in buildings. The external environment may require suitable controls to avoid potential contamination wherever the excipient or in-process material is exposed.

Excipient Properties and Functionality: Excipients are frequently used in those types of drug products for which physical characteristics, such as particle size, may be important. Although the manufacturer of the finished dosage form is primarily responsible for identifying the particular physical characteristics needed, it is also the responsibility of the excipient manufacturer to control excipient manufacturing processes to ensure consistent conformity to excipient specifications. Wherever possible, consideration should be given to the end use of the excipient. This is particularly important if the excipient is a direct component of a sterile drug product or one that is claimed to be pyrogen-free.

Consistency of Manufacture and Change Control: A thorough understanding of the manufacturing process and effective control of change can best ensure consistency of excipient quality from batch to batch. Implementation of changes may also have consequences for registration filings with regulatory agencies.

Changes in excipient manufacturing processes may result in changed physical or chemical properties of the excipient that are evident only during subsequent processing or in the finished dosage form. This is particularly important in the context of the pharmaceutical product approval process where bioequivalence comparisons are made between pivotal, clinical trial batch (*bio batch*) production and commercial scale-up batches. Changes made to the excipient supplied for the commercial product from the excipient supplied for the bio batch should not affect the quality and performance of the commercial drug product. Scale-up of excipients to commercial production may involve several stages, and data may be required to demonstrate consistency between batches through the scale-up process.

Traceability: Traceability of batch-related records to facilitate investigations and retrieval of product is also a key requirement of GMP.

APPLICATION OF GMP PRINCIPLES

It is the responsibility of the excipient manufacturer to designate and document the rationale for the point in the manufacturing process at which appropriate GMP are to be applied. From this point on, appropriate GMP should be applied.

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The manufacturer should apply a level of GMP to each manufacturing stage commensurate with the importance of that step in ensuring product integrity. This may be demonstrated by means of the use of a risk assessment procedure (for example, HACCP, FMEA).

The stringency of GMP in excipient production should increase as the process proceeds from early manufacturing to final stages, purification, and packaging. Physical processing (for example, granulation, coating, or physical manipulation of particle size such as milling or micronizing) as well as chemical processing of excipients should be conducted at least to the standards suggested by this chapter.

It should be recognized that not all intermediates may require testing. An excipient manufacturer should, however, be able to identify critical or key points in the manufacturing process where selective intermediate sampling and testing are necessary in order to monitor process performance.

GENERAL AUDITING CONSIDERATIONS

Audits of an excipient operation will be influenced by the purpose of the audit and the intended use of the excipient. The key stages of a production process should be examined to determine whether the manufacturer controls these steps so that the process performs consistently. Overall, an audit should assess the excipient manufacturer's capability to deliver a product that consistently meets established specifications.

The audit team may consist of engineers, laboratory analysts, purchasing agents, computer experts, maintenance staff, and other personnel as appropriate to the scope and purpose of the audit. External auditors must respect the confidentiality of the manufacturer's processes and other disclosures.

An audit should focus on the quality-critical processing steps that are necessary for producing an excipient that meets established physical and chemical criteria. These steps should be identified and controlled by the excipient manufacturer. Quality-critical processing steps can involve a number of unit operations or unit processes. Quality-critical steps can include, but are not limited to, the following:

- phase changes involving the desired molecule, solvent, inert carrier or vehicle (for example, dissolution, crystallization, evaporation, drying, sublimation, distillation, or absorption),
- phase separation (for example, filtration or centrifugation),
- chemical changes involving the desired molecule (for example, removal or addition of water of hydration, acetylation or formation of a salt),
- adjustments of the solution containing the molecule (for example, pH adjustment),
- precise measurement of added excipient components, in-process solutions, and recycled materials (for example, weighing or volumetric measurements),
- mixing of multiple components,
- changes that occur in surface area, particle size, or batch uniformity (for example, milling, agglomeration, or blending).

AUDIT CHECK POINTS

A good approach for an excipient plant audit is a review of the following areas:

- nonconformities—such as the rejection of a batch that did not meet specifications, customer complaints, return of a product by a customer, or retrieval of a product. The manufacturer should have determined the cause of the nonconformity, prepared a report of the investigation, and initiated and documented subsequent corrective action. Records and documents should be reviewed to ensure that nonconformities are not the result of a poorly developed or inconsistent process;
- customer complaint files—such as reports that some aspect of the product is not entirely suitable for use, because such problems may be caused by impurities or inconsistencies in the excipient manufacturing process;
- change control logs—to ascertain whether the company evaluates its significant changes to decide whether the customer and/or regulatory authority should be notified;
- nonconforming products meeting or Material Review Board documents and/or equivalent records that demonstrate that the disposition of nonconforming product is handled in an appropriate manner by responsible individuals;
- master formula and production records for frequent revisions that may reveal problems in the excipient production process;
- evidence for the presence of unreacted intermediates and solvent residues in the finished excipient;
- materials management systems, to ensure adequate control over nonconforming materials so that they cannot be sold to customers or used in manufacturing without authorization;
- review of a process flow diagram, to aid understanding of the various processing stages. The critical stages and sampling points should be identified as part of the review of the processing records;
- review of contamination control measures.

In evaluating the adequacy of measures taken to prevent contamination and cross-contamination of materials in the process, it is appropriate to consider the following risk factors:

- the type of system (for example, open or closed). Enclosed systems in chemical plants often are not closed when they are being charged and/or when the final product is being emptied. In addition, the same reaction vessels are sometimes used for different reactions;
- the form of the material (for example, wet or dry);
- the stage of processing and use of the equipment and/or area (for example, multipurpose or dedicated);
- continuous versus batch production.

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DOCUMENTATION AND RECORD REVIEW

Documentation required for the early steps in the process need not be as comprehensive as in the latter stages of the process. It is important that a chain of documentation exist and that it be complete when the following is the case:

- the excipient can be identified and quantified for processes where the molecule is produced during the course of the process. For batch production, a theoretical mass balance may also be established with appropriate limits, because deviations from tolerance are a good indicator of a loss of control;
- an impurity or other substance likely to adversely affect the impurity profile or form of the molecule is identified, and subsequent attempts are made to remove it.

As chemical processing proceeds, a chain of documentation should be established that includes the following:

- a documented process,
- the identification of critical processing steps,
- appropriate production records,
- records of initial and subsequent batch numbers,
- records of raw materials used,
- comparison of test results against meaningful standards.

If significant deviations from the normal manufacturing process are recorded, there should be evidence of suitable investigations and a review of the quality of the excipient. Complete documentation should be continued throughout the remainder of the process for quality-critical processing steps until the excipient is packaged and delivered to the end user. The batch should be homogeneous within the manufacturer's specifications. This does not necessitate the final blending of continuous-process material if process controls can demonstrate compliance with specifications throughout the batch.

In order to promote uniformity in excipient GMP inspections, the following basic requirements should be established:

- assignment of a unique batch number to the excipient, enabling it to be traced through manufacture to release and certification,
- suitable controls for the preparation of a batch record for batch processing and/or a production record, log sheet, or other appropriate documentation for continuous processing,
- demonstration that the batch has been prepared using GMP guidelines from the processing point at which excipient GMP have been determined to apply,
- confirmation that the batch is not combined with material from other batches for the purpose of either hiding or diluting an adulterated batch,
- records showing that the batch has been sampled in accordance with a sampling plan that ensures a representative sample of the batch,
- records showing that the batch has been analyzed using scientifically established test methods designed to ensure that the product meets established standards, specifications, and characteristics,
- stability data adequate to support the intended period of use of the excipient; these data can be obtained from historical data, from actual studies on the specific excipient, or from applicable model product studies that can reasonably be expected to simulate the performance of the specific excipient.

⟨1079⟩ GOOD STORAGE AND DISTRIBUTION PRACTICES FOR DRUG PRODUCTS

INTRODUCTION

This general information chapter describes good storage and distribution practices to ensure that drug products (medicines) reach the end user (practitioners and patient/consumers) with quality intact.

In the context of this chapter, the following definitions are used.

SCOPE

Good storage and distribution practices apply to all organizations and individuals involved in any aspect of the storage and distribution of all drug products, including but not limited to the following:

- Manufacturers of drug products for human and veterinary use where manufacturing may involve operations at the application holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Packaging operations by the manufacturer or a designated contractor for the applicant holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer
- Laboratory operations at the manufacturer's or at the contractor's site
- Physician and veterinary offices
- Pharmacies including but not limited to retail, compounding, specialty, mail order, hospital, and nursing home pharmacies
- Importers and exporters of Record

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- Wholesale distributors; distribution companies involved in automobile, rail, sea, and air services
- Third-party logistics providers, freight forwarders, and consolidators
- Health care professional dispensing or administering the drug product to the end user
- Mail distributors including the U.S. Postal Service (USPS) and other shipping services including expedited shipping services

The information is intended to apply to all drug products regardless of environmental storage or distribution requirements.

It is recognized that conceivably there are special cases and many alternative means of fulfilling the intent of this chapter and that these means should be scientifically justified. Although this chapter is not intended to address the storage and distribution of active pharmaceutical ingredients (APIs), excipients, radioactive products, reagents, solvents, medical devices, medical gases, or clinical trial materials for which storage requirements may not yet be defined (e.g., Phase I clinical trial drug products), the general principles outlined here may be useful if applied selectively or comprehensively.

This general information chapter does not supersede or supplant any applicable national, federal, and/or state storage and distribution requirements, or USP monographs. General Chapter ⟨659⟩ *Packaging and Storage Requirements* contains definitions for storage conditions. This chapter is not intended to cover counterfeiting, falsified medicines, drug pedigrees, or other supply chain security, including chain of custody issues.

BACKGROUND INFORMATION

Storage and distribution processes may involve a complex movement of product around the world, differences in documentation and handling requirements, and communication among various entities in the supply chain. The translation of best practices into good storage and distribution meets these challenges and sets forth a state of control.

The good storage and distribution practices described in this chapter should facilitate the movement of drug products throughout a supply chain that is controlled, measured, and analyzed for continuous improvements and should maintain the integrity of the drug product in its packaging during storage and distribution.

RESPONSIBILITIES

The holder of the drug product application, the drug product manufacturer (in the case of many OTCs, where there is no application) and the repackager bear primary responsibility and accountability including but not limited to the following:

- The decision for regulatory submissions, where applicable, relative to the contents of this chapter for the storage and distribution of drug products. If breaches occur in any of the QMS systems and cannot be justified or documented with scientific evidence, the appropriate entity should consider action with the product to ensure the public safety.
- Determining proper storage and handling practices
- Communicating storage and distribution practices through the supply chain
- Drug product stability profiles or the associated stability information from the holder, inclusive of distribution conditions and excursions that may be allowable should they occur. These stability profiles include the approved storage conditions for the shelf life of the drug product and, where appropriate, supporting data for the distribution conditions, if these differ from the storage conditions.
- Appropriate firms, such as an applicant holder, are to convey relevant environmental requirements (e.g., when appropriate, product-specific lifecycle stability data), when needed to support deviations or temperature excursions. If stability data cannot be reviewed or is not shared, an assessment may be needed to consider regulatory review or other appropriate actions (e.g., destruction of product or additional stability testing).
- Recalling the drug product if it is found to be adulterated in any part of the supply chain

However, all organizations along the supply chain bear responsibility for ensuring that they handle drug products within adequate storage and distribution parameters that will not affect the drug product identity, strength, quality, purity, or safety.

Each holder of drug product is responsible and accountable for the receipt from an entity and transfer out of the drug product to the next entity.

LABELING CONSIDERATIONS FOR DRUG PRODUCTS

The environmental requirements for drug product storage conditions should be indicated on the drug product primary container–closure system. If space on the immediate container is too small (e.g., an ampule) or is impractical for the container–closure system (e.g., blister package), this information can be placed on the most immediate container of appropriate size (e.g., carton). Environmental storage conditions and/or environmental warning statements should be evident, securely fixed, and indelible on the outermost container (generally the shipping container).

Products classified as hazardous materials and/or dangerous goods by the U.S. Department of Transportation or other relevant authorities or bodies should be labeled, stored, and handled in accordance with applicable federal/state/local regulations. Drug products classified as controlled substances by the U.S. Drug Enforcement Administration or by individual state requirements should be labeled and handled in accordance with applicable regulations.

Good practices and controls for labeling should provide the receiver with instructions for the correct handling of the drug product upon receipt. When a drug product's storage conditions are not readily available, use the storage conditions described in USP's *Packaging and Storage Requirements* ⟨659⟩.

Product labels with expanded information beyond the single long-term storage temperature ensure ease of transport and use for shippers, distributors, healthcare professionals, and patients. Product labels should clearly define the storage temperature range, and broader distribution or in-use temperature ranges where allowable. Products labeled "Keep in a cold place" or "Do

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not freeze" are subject to interpretation and are discouraged if used without accompanying temperature ranges. USP storage definitions and temperature ranges are defined in *General Notices and Requirements*.

During international transport, the proper language(s) should be used to ensure that handlers understand the requirements set forth on drug product labeling. The use of symbols that are recognized by international organizations is advisable.

Drug products can be transported at temperatures outside of their labeled storage temperatures if stability data and relevant scientific justification demonstrate that product quality is maintained. The length of the stability studies and the storage conditions for a drug product should be sufficient to cover the shipment, distribution, and subsequent use of the drug product. The data gathered from ICH, Q1A R2, accelerated testing or from testing at an ICH intermediate condition may be used to evaluate the effect of short-term excursions outside of the label storage conditions that might occur during storage and/or distribution.

QUALITY MANAGEMENT SYSTEM

Good storage and distribution practices require that entities involved in the storage and/or distribution of drug products maintain a Quality Management System (QMS) that is based on standard quality concepts, includes good manufacturing practice (GMP) in compliance with the appropriate regulatory agency(s), and is complementary to the ICH quality guidances, including ICH *Q10 Pharmaceutical Quality System* and ICH *Q9 Quality Risk Management*. In the context of this chapter, the QMS includes the following management system programs: (1) *Storage Management System*, (2) *Distribution Management System*, (3) *Environmental Management System*, and (4) *Risk Management System*.

The storage and distribution QMS should, at minimum, cover the following elements: corrective and preventive actions (CAPA), change management, deviation/investigation management, and the management review process.

Written agreements (e.g., Quality Agreement, Technical Agreement, Service Level Agreements) should be in place between applicable organizations involved in the drug product supply chain. This means that the originating manufacturer may not be required to hold a Written Agreement with all parties in the supply chain. The use of written agreements ensures clarity and transparency, and delineates the responsibilities of each organization in the supply chain.

Good Documentation Practices

Good documentation practices should be practiced in the QMS. This documentation includes standard operating procedures and corporate policies and standards, as well as protocols and other written documents that delineate the elements of the QMS. The QMS programs should describe events and actions that must be documented as well as the proper verbiage to be used, the copies required, and any other items that will ensure adequate processing of the drug product and prevent delays. The documentation process should use a standard such as a quality manual or other practice and, should include routine assessment for review and update as needed.

Written procedures should ensure that drug products are held in accordance with their labeling instructions and associated regulatory requirements. Procedures should provide the written steps needed to complete a process and ensure consistency and standard outcomes. The following elements should be included: (1) how and when a product should be moved from one transport container/vehicle into another, (2) how products are handled when equipment malfunctions or when there are delays in distribution due to Customs hold, and (3) how to communicate with the necessary parties.

The QMS should require monitoring of processes to demonstrate that a state of control is being maintained, where the set of controls consistently provides assurance of continued process performance and product quality (ICH Q10).

If deviations occur, a nonconformance should be documented, and investigation should be performed and documented as appropriate. The investigative process should determine the root cause(s) of the deviation. For example, the following should be determined: whether the drug product experienced stress, damage, delays, or environmental lapses, or whether there were errors in documentation. The associated supply quality management staff should have final responsibility for approving or rejecting the investigation. The investigation process should be linked to the risk management program to ensure that proper mitigation occurs and preventive measures are put in place.

For example, a written investigation should be performed if the receiving and/or transferring processes result in a drug product being subjected to unacceptable temperature conditions or contamination (e.g., pests, microorganisms, or moisture). Any breach of standard operating procedures should be documented with a risk justification as needed. This information should be forwarded to the appropriate organization responsible for the drug product. The drug product should be quarantined, and final disposition should be based on good science with appropriate evidence to justify the decision(s).

Manufacturers should develop written procedures for recording the security process that confirms container–closure integrity for drug products that require special handling, such as security seals for controlled substances. Returned and salvaged goods records should address how the drug product is assessed through a written procedure. In addition, training on such procedures should be part of the QMS.

Records should be retained for purchases and sales of drug products and should show the date of purchase or supply; the name of the drug product and the amount; the name and address of the supplier or consignee; and the associated lot numbers. These records should allow for the traceability of a drug product in the supply chain.

All records and documents should be maintained in accordance with a traceable records-retention program and should be made available upon request to regulatory agencies. These documents should be approved, signed, and dated by the department responsible for the QMS.

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Storage Management System

STORAGE LOCATIONS AND PROCESSES

It is important that each entity define their appropriate storage locations to ensure that adequate controls are in place. These locations include buildings and facilities for drug product storage (e.g., warehouse, storage or hold area, the original manufacturer's warehouses, contractor warehouses, wholesale distribution warehouses, mail order or retail pharmacy storage area, hospital or nursing home pharmacy storage areas; and border Customs storage areas).

In these locations, two basic processes can occur. First, receiving for storage is the act of bringing a drug product into a facility, while transferring refers to the moving of a drug product internally within a facility or into or out of a vehicle. Second, storing and holding refers to the act of maintaining temporary possession of a drug product in the supply chain process, during which no movement of the product will occur.

STORAGE IN BUILDINGS AND FACILITIES

Drug product storage areas are required to maintain the product temperature between the limits as defined on the product label. Buildings and facilities used for the warehousing, storage, and/or holding of drug products should be of adequate size for their intended use. These facilities should be adequate to prevent overcrowding. The building and facility should be designed to control environmental conditions where necessary and should be made of readily or easily cleanable materials. Sanitation and pest control procedures should be written, indicating frequency of cleaning and the materials and methods used. The pest-control program should ensure the prevention of contamination as well as the safe use of pesticides. Records of all cleaning and pest-control activities should be maintained.

Storage should be orderly and should provide for the segregation of approved, quarantined, rejected, returned, or recalled drug product. If computerized systems are used for the control of storage conditions, the software should be appropriately qualified for its intended purposes. Facilities should have controls that mitigate risks such as fire, water, or explosion. Certain drug products may cause these risks and should be stored accordingly. Storage areas, when not computerized, should be appropriately visually labeled.

Storage facilities themselves, unless thermostatically controlled, cannot be validated; however, they can be qualified via a mapping process. The generator back-up power supply should be qualified.

RECEIVING AND TRANSFERRING DRUG PRODUCTS

Storage of a drug product includes not only the period during which the drug product is held in the manufacturer's storage areas but also time spent at the receiving bay area. When drug products arrive at warehouse loading docks and other arrival areas, they should be transferred as quickly as possible to a designated storage or within a time period that is consistent with the risk and exposure of the product in the receiving area to a designated storage environment to ensure minimal time outside specified storage conditions as described in a written procedure.

Relative to the incoming receipt of drug product, it is recognized that the process of product reaction to ambient conditions begins immediately and may occur quickly (e.g., reach temperature equilibrium within minutes to a few hours depending on details such as the product mass, volume, and packaging density taking into account secondary and tertiary packaging)[1] . Time spent in a transport vehicle is considered to be part of the distribution process and is not a storage location.

Receiving docks should protect drug product deliveries from inclement weather during unloading. Any storage area, including loading and unloading docks for receipt and distribution of drug products, should be clean, cleanable, and free from pests. The incoming receiving area should limit access to authorized persons. Where appropriate, the delivery vehicle/container should be examined before unloading to ensure that adequate protection from contamination was maintained during transit. Deliveries should be examined at receipt in order to check that containers are not damaged and that the consignment corresponds to the order. The results of this examination should be documented.

Areas should be designated to provide an adequate space in which containers of drug products can be cleaned and opened for sampling. If sampling is performed in the receiving area, it should be done in a manner that prevents contamination and cross-contamination and ensures that environmental requirements for the drug product are not breached.

Adequate precautions should be taken to prevent theft and diversion of drug products. Drug products that have been identified as counterfeit should be quarantined to prevent further distribution. The appropriate regulatory agencies should be contacted according to established procedures.

Appropriate delivery records (e.g., as applicable, transport vehicle movement papers, receiving/delivery records, data logging records, temperature recorders and similar devices, bill of lading, house air waybill, master air waybill, etc.) should be reviewed by each receiving entity in the supply chain to determine if the product has been subjected to any transportation delays or other events that could have exposed the product to undesirable conditions. Each entity should ensure that their respective Service Level Agreement documents and supporting documents such as SOPs cover delivery and receiving responsibilities of the transactional parties.

Smoking, eating, and drinking should not be permitted in any storage/hold areas.

REFRIGERATORS AND FREEZERS

Refrigerators and freezers used to store drug products are required to maintain the product temperature between the limits as defined on the product label. Typically, a refrigeration unit specification would be set to 5° with an allowable range of ±3° to store products labeled 2°–8°. Freezer temperatures may vary and typically range from –25° to –10°. Some frozen drug products, however, require lower temperatures, e.g., dry ice or liquid nitrogen temperatures.

[1] JP Emond, *Study for Temperature Sensitive Product: Preliminary Testing*, October 2009, University of Florida.

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Regular operating procedures and maintenance protocols should be in place along with written contractual agreements for all maintenance and evaluation procedures including the following:

1. Items should be stored in the units in a manner that allows adequate air flow to maintain the specified conditions.
2. Units should be positioned in the facility so that they are not subjected to environmental extremes that could affect their performance. If this cannot be prevented, the mapping protocol should include a provision for testing during the anticipated environmental extremes.
3. Large commercial units such as walk-in cold rooms are qualified via a temperature mapping study or other type of qualification process to determine the unit's suitability for storing drug products. A suitable number of temperature-recording devices should be utilized to record temperatures and to provide temperature area maps. Thereafter, the units should be monitored as determined by the results of the mapping study. Refer to the *Temperature Monitoring* section under *Environmental Management System*.
4. Units should utilize recording systems to log and track temperatures. Alarm systems should be an integral part of the monitoring system for both refrigerators and freezers. While automated systems monitor units continuously, manual checks should be performed as appropriate to the validation program. When automated systems are not available, manual systems may be used.

Distribution Management System

Distribution of drug products occurs within a facility or location such as a manufacturer, wholesaler, pharmacy dispensing area, retail site, clinic/hospital/nursing home pharmacy, and the physician's practice. Distribution of drug products occurs as point-to-point movement within the supply chain between distribution facilities via semitrailer trucks, vans, emergency medical service vehicles, industry representatives' automobiles, trains, aircraft, sea vessels, and mail delivery vehicles.

Communication within the supply chain should be coordinated to determine proper timing for drug products to be transported and received, taking into account holiday schedules, weekends, or other forms of interruption. When international distribution is required, alerts should be made in advance and proper language should be used to ensure understanding of the requirements set forth on drug product labeling.

PACKAGING FOR THE DISTRIBUTION AND TRANSPORTATION PROCESSES

Pharmaceutical manufacturers should consider primary, secondary, and tertiary packaging that best protects the drug product during storage and distribution. Package performance testing should be documented as part of a manufacturer's QMS. Several standard test procedures are available for evaluating package performance for factors such as shock, vibration, pressure, compression, and other transit events. Organizations with standard test methods include the following: the American Society for Testing and Materials (ASTM) *Standard Practice for Performance Testing of Shipping Containers and Systems*, and the International Safe Transit Association (ISTA) specifications for various types of transit modes such as less-than-truckload, small package, rail car, and air freight.

It is important to be aware that removal or modification of the original packaging may subject the product to unacceptable conditions.

The packaging (tertiary or thereafter) for the distribution of the drug product should be selected and tested to ensure that product quality is maintained and to protect the contents from the rigors of distribution including environmental or physical damage.

All drug products have storage requirements that may contain specific controls. The container used for transporting the drug product should be qualified on the basis of the labeled conditions of the product as well as anticipated environmental conditions. Consideration should be made for seasonal temperature differences, transportation between hemispheres, and the routes and modes of transport.

The type, size, location, and amount of the temperature stabilizers required to protect the product should be based on documented studies of specific distribution environments including domestic and international lanes, mode(s) of transport, duration, temperature, and other potential environmental exposures or sensitivities that may impact product quality. Transportation container materials such as warm/cold packs and materials used to control temperature conditions should be properly conditioned before use. Barrier protection may be important in helping to determine the position of materials such as gel packs in order to avoid direct contact with the drug product. It should be determined if studies are required to ensure that the dry ice and its vapors do not adversely affect the drug product, including the drug product labeling.

VALIDATION AND THERMAL PERFORMANCE QUALIFICATION FOR TRANSPORT SYSTEMS

Drug product transport systems should be continuously monitored by calibrated monitoring systems, (continuous verification), or shipping systems should be qualified and based on historical data relative to the process. However, it may be acceptable to use product stability data and supply chain risk assessment to justify shipping without either continuous monitoring or qualification of the shipping system.

Operational and performance shipping studies should on a generic level be part of a formal qualification protocol that may use controlled environments or actual field testing, depending on the projected transport channel. These studies should reflect actual load configurations, conditions, and expected environmental extremes. Testing should be performed on both active and passive thermal packaging systems.

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Environmental Management System

While storage and distribution temperature ranges for drug products are labeled on the packaging, relative humidity effects occur over a much longer time frame. The primary container is designed and tested to protect the product from moisture; therefore, humidity monitoring should be considered when a product will be stored in an uncontrolled facility.

TEMPERATURE MONITORING

Environmental conditions are important parameters to consider in the storage and distribution of all drug products and may require monitoring depending on the requirements. When specific storage conditions are required and transportation qualification has not been performed, and in the absence of active or passive containers, environmental recorders or devices should be used to confirm that an acceptable range has been properly maintained during each stage in the supply chain.

Temperature is one of the most important conditions to control, and requirements for each drug product should be based on stability data. Temperatures should be tracked using a monitoring system, and the monitoring devices used should be included in a calibration and/or preventive maintenance program. Environmental monitoring devices should be calibrated for their range of operation. The monitoring devices used should provide an alert mechanism if the preset ranges are breached. The following practices and controls are examples of appropriate measures that should be put in place to ensure environmental control (see also *Monitoring Devices—Time, Temperature, and Humidity* ⟨1118⟩):

- Temperature-monitoring equipment, a monitoring device, a temperature data logger, or other such device that is suitable for its intended purpose should be used.
- An appropriate number of temperature monitors or some other form of recordation or proof of temperature control. Temperature monitor(s) should be used with every distribution process unless another process has been put in place to ensure specified temperature ranges.
- Electronic temperature monitors should be calibrated to National Institute of Standards and Technology (NIST) or other suitable standard.
- Chemical temperature indicators may be used as appropriate.
- Predetermined temperature ranges should be set for all applicable areas, as well as a plan of action in the event of an unacceptable excursion.

TEMPERATURE MAPPING

The basis of any temperature mapping in a temperature controlled space (e.g., facility, vehicle, shipping containers, refrigerator, freezer) is the identification and documentation of a sound rationale used for a given mapping procedure. The temperature variability associated with mapped locations and the level of thermal risk to the product should be defined, unless another process has been put in place to ensure environmental control.

A temperature mapping study should be designed to assess temperature uniformity and stability over time and across a three-dimensional space. Completing a three-dimensional temperature profile should be achieved by measuring points at not less than three dimensional planes in each direction/axis—top-to-bottom, left-to-right, front-to-back, where product will be present.

When temperature mapping is necessary, it should begin with an inspection of the facility, equipment and/or vehicle and should be re-evaluated as appropriate. Environmental mapping also should be performed after any significant modification to the distribution system that could affect drug product temperature.

Facility temperature mapping: The following factors, which may contribute to temperature variability, should be considered during the process of temperature mapping storage locations: (1) size of the space; (2) location of HVAC equipment, space heaters, and air conditioners; (3) sun-facing walls; (4) low ceilings or roofs; (5) geographic location of the area being mapped; (6) airflow inside the storage location; (7) temperature variability outside the storage location; (8) workflow variation and movement of equipment (weekday vs. weekend); (9) loading or storage patterns of product; (10) equipment capabilities (e.g., defrost mode, cycle mode); and (11) SOPs.

The recording of temperatures during the thermal mapping of a warehouse or cold room should be sufficient in time frame to capture workflow variation that may impact air flow and the resulting temperature fluctuation (i.e., a period of one week is recommended for data collection and should capture workflow cycles).

Equipment (container/trailer) temperature mapping: To minimize risk of product exposure to damaging temperatures during transport, dedicated containers/vehicles cargo space should be mapped. When complete fleet mapping (i.e., wholesaler or distributor vehicles) is not realistic or appropriate, minimally at least one container/vehicle from the fleet must be mapped. Thereafter, the following conditions should be considered: (1) SOPs, including loading and unloading procedures; (2) route-specific operation of the temperature control equipment; (3) seasonal effects encountered on expected routes; (4) loading patterns; and (5) transport durations.

When nondedicated (i.e., mail carriers) transport containers/vehicles and equipment are used, they should be designed to minimize the risk of contamination of the product being handled. If environmental mapping of such vehicles is not performed, some other means of control should be in place to ensure that the drug product is adequately protected. Mapping by the shipper may not be necessary if the shipper uses a transport container that is properly insulated and has been previously qualified for the duration of the distribution process by the transport container manufacturer via a mapping study or if drug products are continuously monitored by calibrated monitoring systems (continuous verification).

The vehicle in which drug products are transported should be mapped to determine the appropriate placement of temperature-recording devices and to confirm that the load configuration is not restricting air flow. The following are recommended practices and controls for vehicles that receive and transfer drug products:

1. Transport containers/vehicles and equipment used to store and transport drug products should be suitable for their intended function.

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2. Procedures should be established that describe how to operate, clean, and maintain transport containers/vehicles and equipment used in the storage and distribution of drug products.
3. Transport containers/vehicles should be designed to prevent damage to the drug product, and pharmaceutical manufacturers should collaborate with their transporter to determine contingency response plans for how drug products are handled when equipment malfunction.
4. When drug product must be moved from one transport container/vehicle into another, the proper load configuration should be followed.
5. It should be understood how communication is made to the necessary entities when such transfer occurs.
6. Subcontracted vehicles should be considered in contractual agreements and audits, and documentation should be maintained for their use.

Temperature mapping should account for maximum and minimum loads to capture temperature variability resulting from variations in temperature mass of the payload. Performance of equipment under extreme scenarios including door open, door closed, and simulated equipment failure should be taken into account.

Thermal mapping of vehicles should be representative of the fleet with the intention of capturing variability across the range of vehicles (type of vehicle including non-refrigerated equipment, use, heating and/or cooling system). A periodic requalification program should be documented.

Mapping for both facilities and transportation containers/vehicles should be done in a way that confirms their fitness for operation during periods of expected extreme weather (e.g., summer and winter). Facilities should be mapped under varying operating conditions—ideally during periods of greater variability, accounting for and capturing the result of any seasonal fluctuations of inventory movement, equipment movement, or workflow variation.

The temperature-mapping protocol and associated number of temperature data loggers used to map a three dimensional space should meet the intent of demonstrating three-dimensional uniformity and compliance with product requirements. For both facility and trailer/container temperature mapping, the ambient conditions should be recorded and correlations between ambient conditions and potential thermal risks inside the controlled space should be identified. Drug products should not be stored in areas where a thermal risk has been identified as a result of the temperature mapping. Areas identified as being unsuitable for storage should be clearly labeled as such to ensure that they are not used.

Temperature data loggers should be used for temperature mapping and PQ testing of facilities, equipment, and transportation containers used for storage or transportation of temperature-sensitive medicinal products. Temperature data loggers and any associated software applications should be appropriately validated. Certificates of calibration to an NIST or other international traceable standard should be available for individual monitoring devices.

EXCURSIONS

The mapping process will help determine when excursions could occur and are useful when pharmaceutical manufacturers develop a plan for dealing with them. Alarms should be used to reveal environmental excursions during operations. Temperature excursions for brief periods outside of respective storage label conditions may be acceptable provided stability data and scientific/technical justification exists demonstrating that product quality is not affected (see Health Canada's GUI 0069 entitled, *Guidelines for Temperature Control of Drug Products During Storage and Transportation,* 2011).

MEAN KINETIC TEMPERATURE (MKT) CALCULATION

The MKT is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT may be considered as an isothermal storage temperature that simulates the non-isothermal effects of storage temperature variation. It is not a simple arithmetic mean.

The temperatures used for calculating MKT can be conveniently collected using electronic devices that measure temperatures at frequent intervals (e.g., every 15 minutes). MKT can be calculated directly or the data can be downloaded to a computer for processing. Software to compute the MKT is available commercially.

For dispensing sites, such as pharmacies and hospitals, where the use of such instruments may not be feasible, devices such as high-low thermometers capable of indicating weekly high and low temperatures may be employed. The arithmetic mean of the weekly high and low temperatures is then used in the calculation of MKT. MKT is calculated by the following equation (derived from the Arrhenius equation):

$$T_k = \frac{\Delta H/R}{-ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \ldots + e^{-\Delta H/RT_n}}{n}\right)}$$

where T_k is the mean kinetic temperature; ΔH is the heat of activation, 83.144 kJ · mole^{-1} (unless more accurate information is available from experimental studies); R is the universal gas constant, 8.3144 × 10^{-3} kJ · mole^{-1} · degree^{-1}; T_1 is the value for the temperature recorded during the first time period, e.g., the first week; T_2 is the value for the temperature recorded during the second time period, e.g., second week; and T_n is the value for the temperature recorded during the nth time period, e.g., nth week, n being the total number of storage temperatures recorded during the observation period. [NOTE—All temperatures, T, are absolute temperatures in degrees Kelvin (K).]

MKT DURING STORAGE AND DISTRIBUTION

The holding of a drug may occur as part of storage and distribution practices. Drug products in the distribution supply chain may be held at temperatures outside their labeled storage requirements as determined by an appropriate stability study. Drug

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products stored either in warehouse conditions or in transportation modes may experience excursions from their acceptable temperature ranges. Each product excursion must be evaluated to determine the final product effect. The means of evaluation must be scientifically sound with documented technical justification that the integrity of the drug product has not been affected. One method of analysis for drug product stored outside its respective label storage conditions is the use of an MKT calculation.

Because MKT expresses the cumulative thermal stress a drug product experiences, it is considered an acceptable practice for storage, and it follows that it should be considered for transit excursions in the process of distribution. The calculation must be justified for use with distribution excursions by confirming that the stability limiting characteristic of the product follows first order kinetics over the temperature range encountered. The ICH stability-testing guidelines define MKT as a "single" derived temperature, which, if maintained over a defined period, would afford the same thermal challenge to a pharmaceutical product as would have been experienced over a range of both higher and lower temperatures for an equivalent defined period.

The MKT analysis must be based on good science and should take into account the integrity of the product. The calculated MKT is not sensitive to the impact of excursions that may occur if the baseline is a long period of time such as a storage segment or the entire lifetime of the drug product. For shorter baseline periods of time, such as transport segments, an excursion can have a significant impact on the resulting MKT for that segment; however, this would not necessarily have a significant impact on product quality.

The MKT analysis may be used for storage conditions that have exceeded the acceptable parameters for a drug product, for a short period of time and is not intended to be a measure for long-term storage.

Knowing the MKT for an excursion is useful for evaluating the potential impact on product quality. However, it is also essential to know the upper and lower temperature limits of any excursion. If these extreme temperatures are outside available stability data, it may not be possible to predict the quality impact of the excursion with any confidence regardless of the MKT. Although higher temperatures are given greater weight in the calculation, the calculation of MKT for nonfrozen product that becomes frozen for any amount of time may not result in an acceptable temperature although the product may not be adulterated. At higher temperatures the kinetics of degradation may change or new degradation reactions may occur; at lower temperatures (near freezing) a phase change may occur that is known to have a negative impact on the quality of some drug products (e.g., some proteins and vaccines). For an example of a calculation, see *Pharmaceutical Calculations in Pharmacy Practice* ⟨1160⟩.

Emergency Medical Service Vehicles, Automobiles, and Van Transportation

Road vehicles used to transport drug products (e.g., ambulances and other emergency response vehicles, vans, or automobiles, including those used by sales representatives to transport physicians' samples) should be suitable for their purpose. Monitoring devices should be placed in different areas of the trunk or cabin where the drug product will be positioned during seasonal extremes (e.g., summer and winter). The monitor should be secured so that it is immobile, and there should be no ambiguity about its exact position within the payload so that the monitor is always placed in the same position. Monitoring devices used on or in packages or on containers may also be used. Suitable measures should be taken to maintain the drug product within the allowable limits of the labeled storage requirements. Storage of physician drug product samples by sales representatives is regulated under 21 CFR Part 203.34(b)(4).

Mail Order Pharmacy Distribution

The mailing party is accountable for the appropriate mailing process. Mail distributors including the U.S. Postal Service (USPS) and other shipping services including expedited shipping services are responsible to provide the service contracted.

In the event that the package cannot be delivered as scheduled, the package should be returned to the mailing pharmacy.

Risk Management System

Risk Management System strategies should ensure that each organization's best interests are served by adhering to proper practices, controls, and procedures, including but not limited to the following: the nature of the drug products; distribution requirements on the readable container labeling; exposure to adverse environmental conditions; number of stages/receipts in the supply chain; manufacturer's written instructions; contractors; and drugs at risk from freezing (vaccines, insulin, and biological products) or elevated temperatures (fatty-based suppositories, vaccines, insulin, and biological products).

Examples of risks include the following: (1) vibration that can cause aggregation of some drug products such as proteins and peptide-based drugs; (2) temperature excursions that may lead to phase changes (melting or freezing); (3) loss of container–closure integrity in transit that could cause glass fractures or loss of sterility in sterile drug product containers; and (4) ingress of water or oxygen that could lead to an increase in degradation products. Appropriate firms such as applicant holders are recommended to convey relevant environmental requirements when needed to support deviations or excursions. There may be alternate ways of determining acceptable environmental conditions and these should be documented and justified.

Pharmaceutical manufacturers should ensure that suppliers of drug product transportation are monitored. Auditing transportation firms should be carried out routinely to ensure adequate product handling. The manufacturer's change control system should capture and evaluate changes in logistic factors such as warehouse or receiving areas and vehicle changes.

CONCLUSION

The practices and processes set forth in this general information chapter apply to storage and distribution as part of the life-cycle management of drug products. All involved should ensure the product to its point of use, creating a contiguous supply network that is collaborative and emphasizes preventive measures to protect drug product quality. The increase in global processes coupled with products requiring special environmental controls highlights the need for a strong QM program. QM

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should provide the foundation for maintaining the storage and distribution practices in a continual improvement program and part of an overall management system review by each entity, as appropriate, in the supply chain.

It is equally important to stay current and be ready to change as new solutions evolve. These new technologies should be considered in developing strategies for good distribution practices, controls, and procedures.

GLOSSARY

Adulteration: FDA FDC Act, SEC. 501 (351), A drug or device shall be deemed to be adulterated, if (2)(A) It has been prepared, packed, or held under insanitary conditions it may have been contaminated with filth, or whereby it may have been rendered injurious to health; or (B) the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice to assure that such drug meets the requirements of this Act as to safety and has the identify and strength, and meets the quality and purity characteristics, which it purports or is represented to possess.

Continuous improvement: Recurring activity to increase the ability to fulfill requirements (see *Quality Management Systems—Fundamentals and Vocabulary. ISO Standard 9000:2005*).

Distribution: Refers to elements such as shipping and transportation activities that are associated with the movement and supply of drug products.

Distribution management system: A program that covers the movement, including storage and transportation, of drug products.

Documentation: Recorded information.

Drug products: Medicines, including marketed human and veterinary prescription finished dosage medications, in-process/intermediate/bulk materials, drug product samples, clinical trial materials, over-the-counter products (OTC).

End user: The patient as well as the healthcare provider administering the drug product to the patient.

Environmental management system: A management system that allows for the identification of quality critical environmental aspects (such as temperature, humidity, and/or other environmental factors) for the drug product and ensures that adequate processes to maintain that environment are in place.

Hazardous materials and/or dangerous goods: Any item or chemical which, when being transported or moved, is a risk to public safety or the environment, and is regulated as such under any of the following: Hazardous Materials Regulations (49 CFR 100–180); International Maritime Dangerous Goods Code; Dangerous Goods Regulations of the International Air Transport Association; Technical Instructions of the International Civil Aviation Organization; or the U.S. Air Force Joint Manual, *Preparing Hazardous Materials for Military Air Shipments.*

International Conference on Harmonization (ICH) Guidance for Industry, *Q10 Pharmaceutical Quality System; ICH Q9, Quality Risk Management; and, ICH Q1A R2, Stability Testing of New Drug Substances and Products*: Internationally harmonized documents intended to assist the pharmaceutical industry.

Mean kinetic temperature (MKT): The single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures.

Preventive actions: The measures to eliminate the cause of a potential nonconformity or other undesirable potential situation.

Quality: The physical, chemical, microbiological, biological, bioavailability, and stability attributes that a drug product should maintain in order to be deemed suitable for therapeutic or diagnostic use. In this chapter, the term is also understood to convey the properties of safety, identity, strength, quality, and purity.

Quality management system (QMS): In the context of this chapter, minimally a set of policies, processes, and procedures that enable the identification, measurement, control, and improvement of the distribution and storage of drug product. It is the management system used to direct and control a company with regard to quality (see ICH Q10 model and *Quality System—Fundamentals and Vocabulary, ISO Standard 9000:2005*).

Risk management system: A systematic process used to assess, control, communicate, and review risks to the quality of a drug product across the product lifecycle. Integral to an effective pharmaceutical quality system, it is a systematic and proactive approach to identifying, scientifically evaluating, and controlling potential risks to quality as described in ICH Q10. It facilitates continual improvement of process performance and product quality throughout the product lifecycle. ICH Q9 Quality Risk Management provides principles and examples of tools that can be applied to different aspects of pharmaceutical quality.

Written agreement or contract (commonly referred to as a Quality Agreement, Technical Agreement, Service Level Agreement, or other): A negotiated, documented agreement between the drug product owner and service provider that defines the common understanding about materials or service, quality specifications, responsibilities, guarantees, and communication mechanisms. It can be either legally binding or an information agreement. A Service Level Agreement may also specify the target and minimum level of performance, operation, or other service attributes.

Storage management system: A program that is used to control the storage of drug products.

Supply chain: The continuum of entities spanning the storage and distribution lifecycle of a product to the end user.

Temperature stabilizer: A material or combination of materials that stores and releases thermal energy used to maintain a specified temperature range within an active or passive packaging container or system (e.g., water-, chemical-, or oil-based phase change material, such as carbon dioxide solid/dry ice and liquid nitrogen).

Transport vehicles: Vehicles used in the supply chain including semitrailer trucks, vans, trains, airplanes, sea vessels, and mail delivery vehicles. Other vehicles, when used to transport drug products are included here, such as emergency medical service vehicles and industry representatives' automobiles.

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⟨1079.1⟩ STORAGE AND TRANSPORTATION OF INVESTIGATIONAL DRUG PRODUCTS

INTRODUCTION

Clinical trials are drug studies that are performed to determine if an investigational medicine meets the effectiveness and safety criteria as has been outlined in the protocol for the clinical trial. Investigational drug products (IDPs) are products not commercially available for the indication or dosage being tested; there may be situations when a commercially available product may be used in a clinical trial as a positive control (comparator), a new indication, or a rescue medication. The term IDP is used throughout this document. Where IDP is used, others may be more familiar with the European Union term investigational medicinal product (IMP). The definition of an IMP is provided in European Commission Directive 2001/20/EC, Article 2 (d), as "a pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical trial, including products already with a marketing authorization but used or assembled (formulated or packaged) in a way different from the authorized form, or when used for an unauthorized indication, or when used to gain further information about the authorized form". For the purposes of this chapter, we will consider the two terms as equivalent. The pre-commercial nature of IDPs means the manufacturing ingredients, including active pharmaceutical ingredients (APIs), excipients, the clinical trial dosage, and any associated stability and packaging components may not be as defined as the final approved finished product. The Food and Drug Administration (FDA) current Good Manufacturing Practice regulations for finished drug products apply equally to commercial products and IDPs.

IDP distribution differs from commercial distribution in that the quantities for IDPs are often small (e.g., as little as one or two bottles or unit dose packages) and there are numerous final destinations, such as clinics and remote clinical settings. Another difference from a commercial drug product is the known stability of the IDP that is often a new chemical/molecular entity in the early stages of clinical trials and has not been through the robust stability program of commercial products. The IDP also creates a unique challenge when compared to a commercial product, which is ensuring the proper distribution of every packaging system, because a temperature excursion may jeopardize the entire clinical trial outcome.

SCOPE

Due to global security issues, regulations may vary and/or change for the distribution of drug products and for IDPs as well. Sponsors, clinical trial site personnel, or their designees are responsible for control of IDPs and should maintain an understanding of the current country distribution requirements. This chapter will address the aspects of storage and distribution that are unique to IDPs (e.g., unblinding, comparators, and academic studies); other chapters of interest may be *Monitoring Devices—Time, Temperature, and Humidity* ⟨1118⟩, *Good Storage and Distribution Practices for Drug Products* ⟨1079⟩, and *Good Distribution Practices for Bulk Pharmaceutical Excipients* ⟨1197⟩. This guidance chapter applies to all IDPs, including drug device combinations as well as non-commercial clinical IDPs, but does not apply to medical devices. The non-commercial clinical trial may have limited financial commercial return and is often performed in an academic setting or by a compounding pharmacy with limited industry participation (see *Figure 1*).

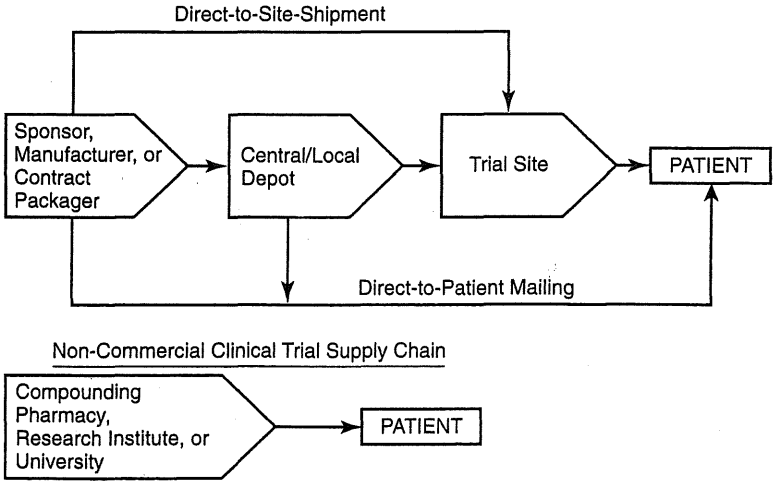

Figure 1. IDP supply chain scheme for commercial and non-commercial clinical trial studies.

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KEY FACTORS FOR IDP DISTRIBUTION

Number of Clinical Trial Sites

The larger the number of clinical trial sites, the greater the risk involved, as this may mean more countries, shipments, and customs challenges. The IDP sponsor company and distributor should be aware of customs requirements in all countries where the IDP is to be distributed. IDPs may be transported from one clinical trial site to another, and the distribution of these IDPs must undergo the same distribution requirements as any other drug products being transported.

Audits

CLINICAL TRIAL SITES

Clinical trial sites must store IDPs in an area that is safe and secure with limited access, and ensure products are maintained at their labeled storage conditions. A site qualification audit will ensure that the sites have adequate space and segregation for both IDPs that have not been dispensed to patients, as well as IDPs that are awaiting return to a sponsor or contractor for final reconciliation and disposition. Depending on the regulatory requirements of the countries where the studies are being conducted, calibrated thermometers or an automated temperature tracking and alarm system should be in place to document site compliance.

DEPOTS AND SUBCONTRACTORS

These places and individuals should follow both the sponsor requirements and associated country regulations in which the study is being conducted.

Available Product Quantities

In early stage development (i.e., Phase 1 and early Phase 2), there are typically fewer APIs and IDPs available. Disruptions in the supply chain will have greater impact and consequences to the timely completion of clinical trials, and distribution delays (especially for biologics) may impact enrollment and study completion.

Expiration/Retest Dates

As the stability profile is being developed during the early phases of development, expiry or re-test date extensions should be anticipated. IDP expiry or re-test dates should follow local regulatory requirements throughout the life of the study. Expiry or re-test dates can be tracked through a validated electronic system, though IDP expiry or re-test dates at global sites or depots may need to be manually updated.

Qualification of Packaging for IDP and Track and Trace

There is no difference in the packaging qualifications for IDPs and commercially available drug products.

Determining what the required environmental conditions of IDP shipments will be during the early stages of a clinical research protocol is of particular importance, as this is the best time to define the robust stability profile of the study drug. Important distribution risk factors to consider include the distance, time, temperature, and the number and type of handoffs that may occur. Packaging that is appropriate to the clinical phase should be used to protect the contents. When that is not possible, the intent is to use qualified components (i.e., components that have been tested to show positive outcomes for maintaining temperatures for a wide variety of products in a wide variety of environmental conditions) to ensure the IDPs inside the shipping containers are controlled and remain at the correct temperature during the journey from warehouse (or depot) to clinical trial site. There are, however, sophisticated track and trace technologies that permit the trial sponsor, packaging contractor, clinical research organization, and the clinical trial site to monitor (track) the IDP along the supply chain.

Performance qualification (PQ) of packaging systems for shipping IDPs should be carried out reflecting the actual load configuration, duration of travel, and expected environmental conditions. There should be no difference in the PQ rational for investigational and commercial drug products because the risks of storage and transport are not related to the marketing authorization. Details of PQ as a strategy for mitigating risks are provided in ⟨1079⟩.

IDP Supply Chain Challenges

One of the larger IDP supply chain challenges is with the number of clinical trial supply shipments that are made in support of clinical trials. In IDP distribution circles, these shipments are referred to as "outbound" shipments. The faster the trial enrolls, the larger the number of shipments. With each shipment we now increase the risk of damage as well as temperature excursions. Each of these would mean shipping additional IDPs to support the trial and added cost to the sponsor company.

Among the challenges for the IDP supply chain are the frequency and size of shipments. These are influenced by such factors as number of patients, frequency of IDP administration to patients, the number and size of patient kits in each shipment, and the expense associated with each shipment.

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Timing

Clinical trial studies that are longer in duration than the original plan may mean more IDP shipments and the need to ensure these extensions continue being in compliance with current good distribution practices. There are instances when IDP inventory remains in the investigational site when enrollment is complete, even though enrolled patients continue to take their remaining treatment. In other instances, IDP may remain in the institution while awaiting return/destruction instructions from the sponsor. The guidelines in this chapter apply to both instances.

Key Global Challenges for IDPs

Global challenges include security, language, culture, tariff codes/taxes, hiring, training, customs, holidays, country-specific import and export requirements, time differences, airline schedules, and documentation. Guidance for such issues can be found in regulatory guidance documents [e.g., FDA Drug Supply Chain Security Act (DSCSA) or International Council for Harmonisation (ICH) Q Series].

IDP ENVIRONMENTAL CONDITIONS

The environmental controls for the distribution of IDPs may be provided for on container labeling, product certificate of analysis, safety data sheet, or other records available for shipping purposes; however, these environmental conditions are not always known at the time of transportation to the trial sites, such as the clinical setting or the patient's home (for home administration). There must be confirmation that the proper steps are taken to ensure IDP supply chain integrity throughout the distribution process. At the point that the IDP is dispensed to the research participant (subject or patient), and is in their custody, the proper handling and storage of the IDP should be explained (in written form or verbally) at the clinical site, in the hospital, or in the participant's home. IDPs that have been dispensed to a patient or subject may not be returned to research stock at the investigator site to be dispensed again. In the case where the IDP is provided to a member of the research team to be dispensed to the participant, but it is not dispensed to the subject or patient, and the chain of custody can be verified to have remained within the study team, the IDP may be received back to the investigational drug service to be dispensed again only if there are standard operating procedures (SOPs) and documentation in place to ensure control of the IDP. Please note that this redispensing guidance may be overruled if the specific protocol or sponsor SOPs or instructions forbids redispensing in any case.

Unblinding

Blinding of IDPs is performed to reduce or eliminate bias in clinical trials and assists in providing randomization across patient populations. In certain circumstances, in order to maintain the safety of research participants, emergency unblinding is necessary. This may be done for an individual dose or a batch/lot. In either case, the unblinded IDP would be separated from the supply of the IDP waiting to be dispensed. Depending upon the reason for the unblinding, the sponsor shall provide instruction regarding return of the IDP or destruction. The impact of unblinding a product can create a need for distribution of a new IDP, either from the manufacturer, depot, or from another clinical trial site. The supply chain partners should have a written procedure establishing responsibilities, procedures, precautions, records, and final destination regarding the unblinding of IDPs to ensure proper handling of this material and avoid the redistribution of IDPs with a broken randomized code.

Comparators

Most IDPs in late Phase 2 and all of Phase 3 clinical trials will be compared to the current standard of care (i.e., commercially available product). This process is generally done using a comparator product that may or may not be currently in the market place in countries where the clinical trial is being conducted. The distribution process for the comparator should follow the shipping requirements on the IDP labeling for that product or from the manufacturer of the product. The IDP provider using comparators should understand and follow the distribution requirements of the comparator to ensure a safe and efficacious product for the clinical trial patient.

Returned IDPs

There should be the same concern for environmental monitoring and supply chain integrity for returned IDPs, as some sponsors may choose to redistribute any returned IDPs that are unopened and undispensed to other clinical trial sites. Returned IDPs intended for redistribution must meet all requirements (i.e., initial release criteria) of the product and associated local regulations. The decision to reuse returned IDPs should be supported by a risk-based assessment.

ASSESSING RISK EARLY IN THE DISTRIBUTION PROCESS

When shipping IDPs to depots and clinical trial sites, using common shipping lanes (routes between the point of origin and the destination point) and within appropriate temperature zones, a risk assessment can identify the probability and the impact missteps in the supply chain. ICH Q9 presents some risk assessment tools that can be used to help organizations to identify their risks and how to address them. Factors such as product category (e.g., narcotics, biologics, radiopharmaceuticals), storage conditions specification (e.g., temperature, light, humidity, vibration), environmental conditions that products will face according to the transportation route (e.g., origin, destiny, and lanes), duration of journey, shipping container (e.g., if

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temperature-controlled, if active or passive), and type of vehicle should be taken into consideration for risk assessment. Organizations should also evaluate if delays along the transportation route or due to custom holds can increase risk rates and should propose strategies to mitigate these risks.

GLOSSARY

Blinding: The process through which one or more parties to a clinical trial are unaware of the treatment assignments. Blinded studies are conducted to prevent the unintentional biases that can affect subject data when treatment assignments are known.

Comparator: An investigational or marketed product (i.e., active control), or placebo, used as a reference in a clinical trial.

Distribution: Refers to elements such as shipping and transportation activities that are associated with the movement and supply of drug products.

Investigational drug product (IDP): Any pharmaceutical product (new product or reference product) or placebo being tested or used as a reference in a clinical trial.

Protocol: The study plan on which a clinical trial is based.

Rescue medication: Medicines identified in the protocol as those that may be administered to the patients when the efficacy of the investigational drug product is not satisfactory, or the effect of the IDP is too great and is likely to cause a hazard to the patient, or to manage an emergency situation.

Standard operating procedure (SOP): Detailed written instructions to achieve uniformity of performance of a specific function. ICH/ISO 9001 required for repeated application of processes that require standardization. All SOPs require quality assurance approval.

Trial site: The location where trial-related activities are actually conducted.

⟨1080⟩ BULK PHARMACEUTICAL EXCIPIENTS—CERTIFICATE OF ANALYSIS

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1. INTRODUCTION

1.1 Purpose

This general information chapter is derived from the *Certificate of Analysis Guide for Bulk Pharmaceutical Excipients*, prepared by the International Pharmaceutical Excipients Council of the Americas (IPEC-Americas) and is meant to serve as a guide for the preparation and appropriate use of a Certificate of Analysis (COA) for pharmaceutical excipients. The goals are to standardize the content and suggest a format for COAs for excipients, and to clearly define the roles and responsibilities for the excipient manufacturer and distributor. The detailed definitions and discussions are intended to establish a uniform approach. By providing this foundation for mutual understanding, the COA will serve as an important element of the overall supply chain controls needed to provide the user with assurance of excipient conformance to specification and its suitability for use in pharmaceuticals.

The chapter is divided into several parts. The first part provides background discussion necessary for the design and required elements of a COA. The section *4. COA Content* is provided to show the format and placement of information in the COA. This is followed by a detailed discussion to ensure that the purpose and meaning of the specific information contained in the COA is understood. For a list of terms used in this informational chapter and their definitions, see the *Glossary*.

1.2 Scope

This chapter is applicable to excipients used in the manufacture of pharmaceutical products.

1.3 Principles Adopted

As an international guide, this informational chapter cannot specify all national legal requirements or cover in detail the particular characteristics of every excipient. When considering the use of this chapter, manufacturers, distributors, and users should consider how it may apply to that specific organization's product. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes. The terminology "should" and "it is recommended" do not necessarily mean "must" in the application of this chapter.

2. GENERAL GUIDANCE

The COA is a legal document that certifies the quality of the excipient and demonstrates that the batch conforms to the defined specifications, has been manufactured under recognized principles of good manufacturing practices (GMPs), and is suitable for use in pharmaceuticals. It should not be used in lieu of appropriate qualification of the supplier (*1*).

A COA for excipients should be prepared and issued by the company responsible for the material, following the general guidelines discussed below. It is expected that a complete and accurate COA is provided to the user for each batch and/or delivery of excipient. When analysis is performed by a distributor, the distributor should issue a COA to the user for any analysis that was performed by or on behalf of the distributor. In such cases, industry best practice is for the distributor to provide the user with the original manufacturer's COA and the distributor's COA.

Identification testing by the excipient manufacturer is not a regulatory requirement. The excipient manufacturer is not required to perform identity tests if they have process controls in place that together with testing ensure the identity of the excipient.

3. DESIGN AND REQUIRED ELEMENTS OF A COA

The elements of a COA listed below are included in *4. COA Content*. The excipient supplier may organize the elements on the COA at their discretion; however, the sections in this chapter have been designed to present the required and optional information in a logical manner.

The original manufacturer and manufacturing site should be identified if different from the supplier and supplier location. The intent is to enable the user to ensure that a change in manufacturing location has not occurred without their knowledge. It is essential that the manufacturer be known to the user. To protect confidentiality through the supply chain, the use of codes for manufacturers and manufacturing sites on the COA is acceptable as long as the user can link the code to the manufacturer and site of manufacture. The identity of the excipient should be definitively established by stating the compendial name and trade name (if any), the grade of the material, and the applicable compendial designations on the COA.

A batch number or other means of uniquely identifying the material quantity covered by the COA, and information relating specifically to it, are typically included in a body section (see *4.2 Body*). Unique identification of the excipient links the COA to the relevant specification and is traceable to a specified batch. The date of manufacture and, if applicable, the expiration date, recommended retest date, or other relevant statement regarding the stability of the excipient is typically included in this section (a detailed discussion of dates on the COA is contained in *6. Establishing Dates on a COA*). User-required information could also be included here.

The actual test results applicable to the quantity of material covered by the COA are included in *Appendix 1: Model COA*. Preferably, the acceptance criteria and test results are included for each characteristic listed. Test method designation and acceptance criteria may be communicated to the customer by reference to other controlled documents, e.g., sales specifications. Reporting of actual data and observations is recommended rather than nonspecific "passes" or "conforms" statements, unless the test is qualitative or this is the acceptance criteria as listed in a compendium or other specification.

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If the reported results are not derived from sampling the finished excipient batch, it should be noted on the analysis section of the COA (see *7.2 Data versus Conformance* for a detailed discussion of such considerations). In such cases, alternative options for the origin of test results other than quality control laboratory testing include, for example (*2*):

- In-process testing
- Continuous monitoring of an attribute or variable and application of appropriate statistical process control (SPC) methods

It may be acceptable not to perform a test when the test attribute cannot be present or cannot fail to meet acceptance criteria, e.g., limited by upstream controls that involve measurement of an impurity to ensure it does not enter or form in the process. Not performing a specified test should be supported by a suitable documented rationale based on a documented risk assessment.

The section *4.3 Certification and Compliance Statements* is used to list various statements that may be required, depending on the excipient and agreed to user requirements. Any declaration by the supplier as to compliance with compendial and/or other regulatory requirements is typically included in this section.

The basis for COA approval should appear on the COA (see *8. Use of Electronically Generated COAs*).

4. COA CONTENT

The following information should appear on the COA or by reference. It is important that all pages of the COA are numbered and include the total number of pages for document control, to assure the customer that all pages of the COA are present. See *Appendix 1: Model COA* for an example.

4.1 Identifying Information

- Title "Certificate of Analysis"
- Identity and address of original manufacturing site: name or other suitable identifier that is unique to the manufacturer and site (e.g., code)
- Responsible organization that issues the COA, address, and contact information (if different from the original manufacturer)
- Name (compendial or chemical) and compendial designation, as applicable
- Grade
- Trade name
- Batch number

4.2 Body

- Date of manufacture
- Unique identifier to the excipient specification
- Expiration or retest date (as applicable) or stability statement
- Specification
  - Test name
  - Reference to the test method
  - Acceptance criteria
- Analysis
  - Test results based on the finished excipient sample, or
  - Alternative test results, as appropriate (see *7.3 Alternatives to Excipient Testing*)
  - Date retested (if appropriate)

4.3 Certification and Compliance Statements

- Standard of GMP applied (e.g., IPEC-PQG Excipient, ICH Q7, NSF/IPEC/ANSI 363, EXCiPACT™)
- Additional compliance statements and applicable references to standards
- Potential to meet additional compendial standards
- Content listing and grade of ingredients (if a mixture)
- Customer specified information

4.4 Authorization

- Identity of authorized individual for approval or electronic signature statement
- Date of approval or suitable alternative
- Page number (i.e., 1 of X pages)

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5. REQUIREMENTS FOR COMPENDIAL DESIGNATION

For a supplier to claim a compendial grade on the COA for an excipient, there are two requirements to be met. The first requirement is that the excipient is manufactured according to recognized principles of GMPs (see *General Notices, 3.10 Applicability of Standards*). The second requirement is that the excipient meets all of the acceptance criteria contained in the appropriate compendial monograph, unless its difference is stated on its label, as defined under *General Notices, 3.20 Indicating Conformance*. These expectations remain in effect until its expiration or recommended retest date, when stored according to the manufacturer's recommendations in the manufacturer's original unopened container.

Every compendial article must be so constituted that when examined in accordance with these assay and test procedures, it meets all the requirements in the monograph defining it, as well as meeting any provisions of the *General Notices*, general chapters, or rules, as applicable. However, it is not to be inferred that application of every analytical procedure in the monograph to samples from every production batch is necessarily a prerequisite for assuring compliance with compendial standards before the batch is released for distribution. Data derived from in-process testing or continuous monitoring of an attribute with SPC may be used. With appropriate scientific justification, analytical methods that are equivalent or better (i.e., more accurate, more precise, etc.) to that which appears in the monograph may be substituted by the supplier when judging compliance of the batch with the compendial standards (see *7. Reporting of Data*).

6. ESTABLISHING DATES ON A COA

6.1 General Guidance

In reporting dates on COAs for excipients, it is important that a clear and unambiguous format be used to prevent possible misinterpretation. To accomplish this, it is recommended that the name of the month be used to designate the month (it may be abbreviated), rather than a numerical representation. It is also recommended that the year include all four digits (i.e., Jan. 1, 2010, or 1 Jan. 2010, etc.).

6.2 Date of Manufacture

The date of manufacture should be clearly defined by the original manufacturer and consistently applied for the particular excipient and process based on established policies and procedures.

It is important to note that while repackaging operations are to conform to GMP requirements, repackaging alone is not considered a processing step that can be used in determining the date of manufacture. To provide traceability for a specific excipient batch, other dates may be required in addition to the date of manufacture, to reflect additional steps such as repackaging.

6.3 Expiration Date and Recommended Retest Date

The stability of excipients may be an important factor in the stability of the finished pharmaceutical dosage forms that contain them. Therefore, it is important that the COA indicates stability of the excipient either by reporting the expiration date and/or the recommended retest date. This provides users with key information concerning the usability of the excipient in the period between the date of manufacture and the use of the excipient by the user.

Appropriate expiration and/or recommended retest dates for excipients should be established from the results of a documented stability-testing program or from documented historical data. Expiration and recommended retest dates should not be reported by a supplier without sufficient stability data or product history to support the assigned dates. Where the excipient is repackaged, the effect of this operation and the new packaging materials on the expiry or retest date should be evaluated to determine if such dates need to be changed.

The expiration date of an excipient cannot be extended. The retest date for an excipient is the date indicated by the supplier after which the excipient should be reevaluated to ensure continued compliance with appropriate specifications. An excipient retest date may be extended based upon appropriate testing. The reevaluation of the excipient may include physical inspection and/or appropriate chemical, physical, or microbiological testing.

It is acceptable to report both an expiration date and a recommended retest date on the COA for excipients, if applicable.

If stability data (*3*) are not available for an excipient, then an appropriate statement should be included on or with the COA to indicate what is known about the stability of the material, and/or whether stability studies are in progress.

6.4 Date Retested

If retesting is performed by an excipient supplier (as noted in *6.3 Expiration Date and Recommended Retest Date*), and the results are used by the supplier to extend the length of time that the material may be used, then the date retested should also be reported, preferably on the COA, but alternative communication means are acceptable. The specific tests that were subject to retesting should be clearly identified, and the results obtained upon retesting should be reported. After retesting, a new recommended retest date should be reported on the COA.

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6.5 Additional Dates

Other dates may appear on a COA, if desired by the excipient supplier or requested by the user. Examples include the release date, shipping date, date of testing, and date the COA was printed or approved. Any additional dates that appear on a COA for excipients should include a clear indication of what the date represents.

7. REPORTING OF DATA

7.1 General Guidance

For the excipients listed in *USP–NF*, the product specifications are set by the supplier to include all attributes listed in the monograph. It is not required that analysis of all specification attributes be made on each lot. However, sufficient analysis and evidence of process stability should exist to ensure that the batch meets all specifications before it is released (see *7.3 Alternatives to Excipient Testing*). Periodic testing of all attributes should be performed to confirm continuing compliance. All the attributes should be verified at an appropriate frequency.

General Notices, 6.30. Alternative and Harmonized Methods and Procedures allows the use of alternative methods and/or procedures if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, and in some other aspects of the procedure performance and handling, and give equivalent or better results.

For excipients that are not included in *USP–NF*, specifications should be set by the supplier to ensure that the quality of the material is maintained on a continuing basis and reflects both the inherent properties of the excipient and its manufacturing process. Specification methods should be demonstrated to provide accurate, reproducible, and repeatable results for the characteristic(s) being tested.

7.2 Data versus Conformance

Finished excipient tests are often performed on bulk excipient after all manufacturing processes are complete but prior to packaging. "Where an in-process or bulk excipient test result is traceable to the finished excipient material, such a test result can be reported on the COA" (*2*). When a compendial or specification test is not performed on the excipient batch, in-process, bulk, or package, this should be indicated on the COA. Typical statements or information in lieu of data may include the following:

- "Conforms"
- "If tested will meet compendial requirements"
- A footnote to indicate the last measurement or other suitable practice

Measurements reported on a COA can be derived from:

1. Testing a representative sample from the finished excipient batch
2. In-process testing of a representative sample where the attribute remains unaffected by further routine processing
3. Continuous monitoring of an attribute in combination with SPCs

Where number 2 or number 3 apply, the technique for how the test result was obtained should be described.

Some attributes [e.g., bovine spongiform encephalopathy (BSE)/transmissible spongiform encephalopathy (TSE)], *Residual Solvents* ⟨467⟩, *Elemental Impurities—Limits* ⟨232⟩, and/or genetically modified organism (GMO) statements may not be reported on the COA and may be provided separately.

7.3 Alternatives to Excipient Testing

For excipients used in drugs sold in the United States, if an excipient attribute "has required criteria, there must be some measurement or test of the material in each lot to ensure that the criteria are met. This may be a measurement from a surrogate test, from in-process control data, or from testing or measurement of the finished material in each lot. Conversely, FDA representatives believe that an approach, which allows for skip testing based on a satisfactory product quality history alone is not acceptable from a cGMP standpoint because such an approach does not adequately verify that each lot meets all of its specifications" (*2*).

It is noted that ICH Q6A allows for periodic/skip lot testing of the drug product and drug substance (*4*).

Results from in-process testing can also be used to replace testing on the finished excipient. "To ensure that a lot of excipient material complies with its required properties, it is acceptable to rely on tests or measurements conducted on samples of material taken at an in-process stage of production, provided that the in-process material will not be affected by subsequent processing or holding with respect to the attributes being verified. There should be justification that test results or measurements, or product performance characteristics, do not change from the in-process stage to the finished product" (*2*).

7.3.1 DOCUMENTATION

The supplier of an excipient should develop and maintain documentation that outlines the process control systems and validation data that justify the use of alternatives to finished excipient testing. This documentation should also include procedures for handling the impact of significant changes on the testing program (see *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩).

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7.3.2 EXAMPLES

See *Appendix 2: Alternatives to Excipient Testing—Examples*.

8. USE OF ELECTRONICALLY GENERATED COAs

COAs issued from computer systems without a handwritten signature are commonplace and are acceptable, provided the appropriate controls are in place. The following considerations should be met:

- Access to the computer system for COA management; entering and editing of data should be limited to authorized personnel
- Authentication by username and password as well as the change of each individual password at an appropriate frequency should be required
- Confirmation of the integrity and accuracy of the information stored in the system and transferred to the printed record should be completed during implementation and then periodically verified thereafter
- Data entered into a computer system from which information is extracted for a COA and changes made thereafter should be accompanied by time- and date-stamped audit trails

With these criteria met, the issuance of electronically generated COAs is acceptable, provided the COA includes contact information.

9. DISTRIBUTOR INFORMATION

Distributors provide excipients and associated services such as:

- Make excipient available in the manufacturer's unopened original package (pass through)
- Repackage excipients from bulk quantities
- Purchase excipients for repackaging under a different label

A distributor should not change the original title and data of the COA or other quality documents. Whenever possible, the original manufacturer's documentation should be used, or transcription of data should be verified.

It is expected that the distributor will have the appropriate level of GMP in place (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

GLOSSARY

Acceptance criteria: Numerical limits, ranges, or other suitable measures of acceptance for test results.

Batch (lot): A specific quantity of material produced in a process or series of processes so that batch can be expected to have uniform character and quality within specified limits. In the case of continuous processes, a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval.

Batch (lot) number: A unique combination of numbers, letters, and/or symbols that identifies a batch and from which the production and distribution history can be determined.

Batch process: A manufacturing process or processing step that produces the excipient from a discrete supply of raw material that is present before the completion of the reaction.

Certificate of analysis (COA): A document listing the test methods, specification, and results of testing a representative sample from the batch to be delivered.

Chemical property: A quality attribute that is measured by chemical or physicochemical test methods.

Continuous process or processing: A process that continually produces the excipient from a continuing supply of raw material.

Contract facility: An internal or external facility that provides services to the manufacturer or distributor of an excipient. These can include, but are not limited to, the following: manufacturing facilities, laboratories, repackaging facilities (including labeling), and warehouses.

Date of manufacture: A date indicating the completion of the final manufacturing process (as defined by the supplier for the particular excipient and process).

Date retested: The date when retesting is performed by an excipient supplier to extend the length of time that the material may be used.

Distributor: All parties in the distribution/supply chain starting from the point at which an excipient is transferred outside the control of the original manufacturer's material management system including parties involved in trade and distribution, (re)processors, (re)packagers, transport and warehousing companies, forwarding agents, brokers, traders, and suppliers other than the original manufacturer.

Excipient: Substances other than the drug substance that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.

Expiry (expiration) date: The date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

Impurity: Any component of an excipient that is not the intended chemical entity but is present as a consequence of the raw materials, excipient manufacturing process, or excipient degradation.

Original manufacturer or manufacturer: Person or company manufacturing a material to the stage at which it is designated as a pharmaceutical starting material.

Packaging: The container and its components that hold the excipient for storage and transport to the customer.

Physical property: A quality attribute that can be measured solely by physical means.

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Process: The combination of operating steps (including synthesis, isolation, purification, packaging, etc.) that produce the finished excipient.

Process capability index (C_p): A statistical measurement that can be used to assess whether or not the process is adequate to meet specifications. A state of statistical control can be said to exist if the random variation in test results for a process attribute is such that the calculated process capability is >1.33 (see *Appendix 2: Alternatives to Excipient Testing—Examples* for further definition).

Process step: A documented instruction to the pharmaceutical excipient manufacturing personnel directing that an operation be done.

Processing: Operations to change product characteristics by mainly physical treatment through, e.g., milling, sieving, distilling, filtration, blending.

Repackaging: The action of changing the packaging of the material.

Retest date: The date when a specific batch of material must be reexamined to ensure that it is still suitable for use.

Skip lot (periodic) testing: The performance of specified tests at release on preselected batches and/or at predetermined intervals, rather than on a batch-to-batch basis, with the understanding that those batches not tested still must meet all the acceptance criteria established for that product. This represents a less than full schedule of testing and therefore should be justified, presented to, and approved by the regulatory authority before implementation. When tested, any failure of the starting material to meet the acceptance criteria established for the skip lot (periodic) test should be handled by proper notification of the appropriate regulatory authority or authorities. If these data demonstrate a need to restore routine testing, then batch-by-batch release testing should be reinstated.

Significant change: Any change that has the potential to alter an excipient's physical, chemical, or microbiological property from the norm, and/or that may alter the excipient's performance in the dosage form.

Site: A defined location of the equipment in which the excipient is manufactured. It may be within a larger facility. A change in site may be to a different part of the existing facility, but in a different operational area, or to a remote facility including a contract manufacturer.

Specification: A list of tests; references to analytical procedures; and pre-established numerical limits, ranges, or other criteria for the tests described for a material, that the material is required to meet.

Stable process: A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP specific and customer specific) and is thus acceptable for its intended use.

Stability: Continued conformance of the excipient to its specifications.

Supplier: Person or company providing pharmaceutical starting materials on request. Suppliers may be distributors, manufacturers, traders, etc.

Supply chain: All steps in the entire chain of distribution starting from the point at which an excipient is transferred outside the control of the original manufacturer's material, to a management system downstream, to the final user of the excipient.

User: A party who utilizes an excipient in the manufacture of a drug product or another excipient.

APPENDICES

Appendix 1: Model COA

The following sample COA is provided to illustrate the principles discussed in the guide and is not meant to be prescriptive.

Certificate of Analysis (sample tests, limits, and statements are for demonstration purposes)

Supplier Company Name			
Supplier Company Address			
Manufacturing Location			
Name of Manufacturer (if different from Supplier)		Phone: **xxx-xxx-xxxx**	
Manufacturing Site Address		Fax: **xxx-xxx-xxxx**	
Product: **Trade Name and Descriptor or Common Name**			
Grade: **Grade Designation**		Customer Code: **xxxxxx** (if applicable)	
Batch Number: **xxxxxx**		Date of Manufacture: **dd/mmm/yyyy**	
Recommended Retest Date: *<time from date of manufacture>*			
Compendial Name and Listing: *USP–NF, Ph.Eur., JP,* or *JPE* (List multiple names and designations if nomenclature is different in each compendium)			
Test Results (sample tests & limits for demonstration purposes)			
Test	**Test Method**	**Specification**	**Results**
Appearance	Visual examination	White granular powder	Complies
Foreign matter	Visual examination	Free from visible contamination	Complies
Identification—JPE	Tests A–C	Pass	Complies
Clarity and color	*JPE*	Clear and colorless	Complies

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Certificate of Analysis (sample tests, limits, and statements are for demonstration purposes) *(continued)*

pH (x% solution)	*USP*	5.0–7.0	#.#
Residue on ignition	*JPE*	NMT 1.0% (450°–550°C)	#.# %
Viscosity (x% solution)	*Ph.Eur.*	4.0–7.0 mPa-s (@20°C)	#.# mPa-s
Water insoluble sub.	*USP*	NMT 0.1%	#.# %
Loss on drying (110°C)	*USP*	NMT 5.0%	#.# %
Loss on drying (105°C)	*JPE*	NMT 6.0%	#.# %
Particle size	Supplier method #	99.5% <150 µm	####
Additional Information (sample tests & limits for demonstration purposes)			
Heavy metals	*JPE*	NMT 10 ppm (as Pb)	NMT 10 ppm[a]
Arsenic	*JPE*	NMT 2 ppm	NMT 2 ppm[b]

[a] This test is performed in-process on each batch and the material has been shown not to change in the finished excipient sample.
[b] This test is performed quarterly based on process validation.

The following sample COA is provided to illustrate the principles discussed in the guide and is not meant to be prescriptive.

Supplier Company Name
Supplier Company Address
Product: **Trade Name and Descriptor or Common Name**
Grade: **Grade Designation**
Batch Number: **xxxxxxx**
Certification and Compliance Statements
GMP compliance: This batch of <***Trade Name***> has been manufactured using excipient GMP.
Compendial standards: This batch of <***Trade Name***> complies with all of the current requirements listed in the *United States Pharmacopeia* (*USP*), the *European Pharmacopoeia* (*Ph.Eur.*), and the *Japanese Pharmaceutical Excipients* (*JPE*).
Other certification statements: Any other type of certification e.g., Residual Solvents, GMO derived, or customer-specific information, should be listed here. These may vary depending on regional regulatory requirements, specific GMP issues, and customer desired information, based on their use of the excipient.
Identity of authorized individual for approval: xxxxxxxxxxxxxxxxxxxxxxxx
Title
Date of approval: dd/mmm/yyyy
This COA was released from a controlled electronic document management system.

Appendix 2: Alternatives to Excipient Testing—Examples

The following are examples of situations where alternatives to finished excipient testing might be justified. These are not the only situations where a sound technical basis can be demonstrated, neither are they examples of situations where alternatives to finished excipient testing will always be appropriate.

- An impurity, byproduct, or unreacted raw material could not be present in the product because the raw materials and chemical reactions used could not contain or generate it above the specified limits.
- The process capability index (*Cp*) for the relevant attribute is high and thus indicates a stable process. Statistical analysis of the reduced frequency data should show that the property remains stable and within specifications. A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated, by appropriate means, to show a level of variability that consistently meets all aspects of the stated specification (both pharmacopeia and user specific) and is thus acceptable for its intended use. For continuous processing, it is also important to demonstrate that the material has been produced under conditions where the process has achieved a form of "steady state", i.e., minimal operator intervention and the in-process attributes have been stabilized.
- For a continuous process, the in-process analyses show that the property that is determined at a reduced frequency is stable and within specification. Repeating the test on each batch would be redundant.
- An analysis of an attribute that is determined on every batch in process has been shown to provide assurance that the final test requirement can be met. Such data can be used to support testing the finished excipient at reduced frequency.

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⟨1084⟩ GLYCOPROTEIN AND GLYCAN ANALYSIS—GENERAL CONSIDERATIONS

OVERVIEW

A number of glycoprotein drugs have been developed as a result of advances in biotechnology, and many naturally derived protein drugs possess complex glycan structures. Glycosylation, a posttranslational modification of these proteins, can play an important role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of these agents. The two main types of protein glycosylation are *N*-glycosylation and *O*-glycosylation. Unlike transcription and translation, glycosylation is not a template-driven process; therefore variability in the glycosylation pattern of a protein can arise, caused by different sources or different manufacturing processes. Differences in this pattern are known to affect biological activity. Glycosylation patterns may therefore be an important set of attributes that arise in characterizing a candidate glycoprotein intended for therapeutic use and in ensuring its stability and quality.

The first part of this chapter provides a brief introduction to glycobiology and describes the complexity of glycan structures. The subsequent parts provide flow charts and a series of general analytical strategies that can be used to characterize glycoprotein glycans by means of the following:

1. Direct analysis of glycoproteins; and
2. Analysis of released nonderivatized or derivatized glycans by various methods of chromatographic and electrophoretic separation and mass spectrometry (MS).

Different approaches to analyzing monosaccharides are described at the end of the chapter.

For selected analytical methods, this chapter cross-references other USP chapters, particularly those relating to biotechnology-derived articles (see chapters *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩).

PROTEIN GLYCOSYLATION

Most proteins in eukaryotic cells undergo glycosylation and other posttranslational modifications before being trafficked to lysosomes, becoming membrane bound at the cell surface, or being secreted. Glycosylation varies significantly from cell to cell, tissue to tissue, and species to species because of the varying expression of hundreds of glycosyltransferases and glycosidases located throughout the Golgi apparatus and endoplasmic reticulum (ER). Four main types of enzymatic glycosylation are found in proteins:

1. *N*-Glycosylation, which involves the initial transfer of oligosaccharides to the nitrogen on the terminal amide group of asparagine and their subsequent processing and modification to a series of glycan chains;
2. *O*-Glycosylation, which in general involves the initial transfer of monosaccharides to the hydroxyl groups of serine and threonine and subsequent elongation and branching of the saccharide chain by the addition of monosaccharides;
3. Glycosylphosphatidylinositol (GPI) anchor, which is a glycolipid linked to the *C*-terminus of a protein; and
4. *C*-Glycosylation, which involves the formation of a carbon–carbon bond between the C2 carbon of the indole ring of tryptophan and the C1 carbon of an α-mannopyranosyl residue.

Any given protein may contain multiple *N*-, *O*-, or *C*-glycosylations, but not more than one GPI anchor. A nonenzymatic addition of saccharides, called glycation, can occur when proteins are mixed with reducing sugars via a complex series of reactions. The two protein glycosylation types that are generally of concern and that are analyzed in glycoprotein drug substances are *N*- and *O*-glycosylation. Each of these is discussed below.

N-Glycosylation

The biosynthesis of *N*-glycans in glycoproteins can be described as a four-step process:

1. Lipid-linked glycan chain initiation and elongation;
2. Transfer of oligosaccharide to the protein or nascent polypeptide chain;
3. Processing of the *N*-glycan chain by removal of specific glucose and mannose residues; and
4. Modification of the *N*-glycan chain by the addition of residues to the nonreducing ends of the glycan chain.

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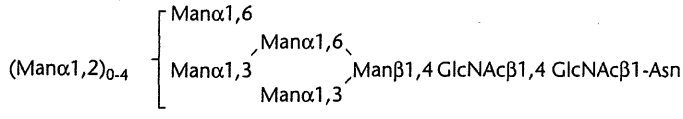
The consensus amino acid sequence for *N*-glycosylation is Asn–Xaa–Thr/Ser (where Xaa is any amino acid other than proline). Overall, only about two-thirds of all potential sequences, termed sequons, are glycosylated, and currently there is no method to predict which sequon will be glycosylated. The role of protein *N*-glycosylation is usually protein trafficking and secretion.

N-glycans can be categorized as high-mannose, hybrid, or complex, depending on the extent of processing *(Figure 1)*. High-mannose structures (*Appendix 1*) lack galactose or *N*-acetylglucosamine (GlcNAc) residues in the antennae, branches at the distal end of the chain. In hybrid structures, both substituted GlcNAc residues and terminal mannose residues are present in the antennae, whereas complex structures have both α1,6- and α1,3-mannose residues substituted with GlcNAc moieties. Hybrid and complex glycans can exist with two or more branches, frequently termed antennae; such glycans are therefore often termed, for example, biantennary, triantennary, or tetraantennary. Both monoantennary and pentaantennary *N*-glycans are also known to exist. In complex glycans, antennae frequently carry terminal sialic acid (neuraminic acid) residues. Sialylation has been shown to have a great effect on both the pharmacokinetics and the pharmacodynamics of many therapeutic glycoproteins.

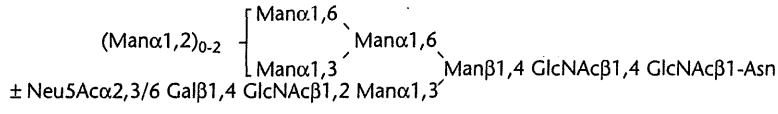
Common type of asparagine (Asn) linked glycans (*N*-glycans).

Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid

High mannose type

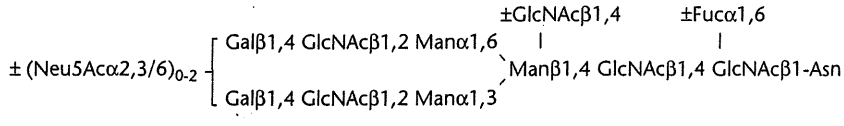


Hybrid type

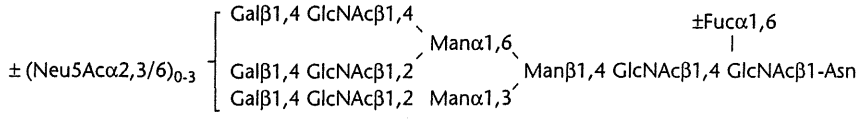


Complex types

Bi-antennary



Tri-antennary



Tetra-antennary

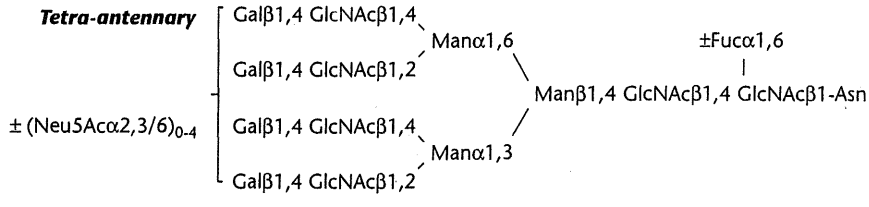


Figure 1. Common types of *N*-glycans. For abbreviations, see *Appendix 1*.

O-Glycosylation

O-Glycan chains are built up sequentially via an initial GalNAc residue linked to serine, threonine, and tyrosine, as well as to the less common amino acids hydroxyproline and hydroxylysine. Multiple glycan core structures are known. The sequence and isomeric linkage of monosaccharides show greater variety than that in *N*-glycans, and at least eight different types have been identified *(Figure 2)*. Although no consensus amino acid sequence for *O*-glycosylation has been determined, glycosylation is usually favored by the presence of proline one residue before or three residues after the glycosylation site and the absence of charged amino acid residues proximal to serine or threonine. The disaccharide unit *N*-acetyllactosamine, Galβ1,4GlcNAc, is the

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most common chain extension. Additional modifications, including terminal capping of Gal with sialic acid and fucosylation along the chain, are also frequent.

O-Glycosylation can occur in cluster form, the mucin type, which usually forms part of the cell surface extracellular matrix or secreted glycoproteins. Other *O*-glycosylation, such as *O*-GlcNAc, is found on many nucleocytoplasmic proteins; and *O*-Man–linked glycosylation is found in some muscular and neural glycoproteins and in yeast. *O*-Fuc– and *O*-Glc–linked glycosylation types are found on many epidermal growth factor-like proteins that are associated with the Notch signaling pathway.

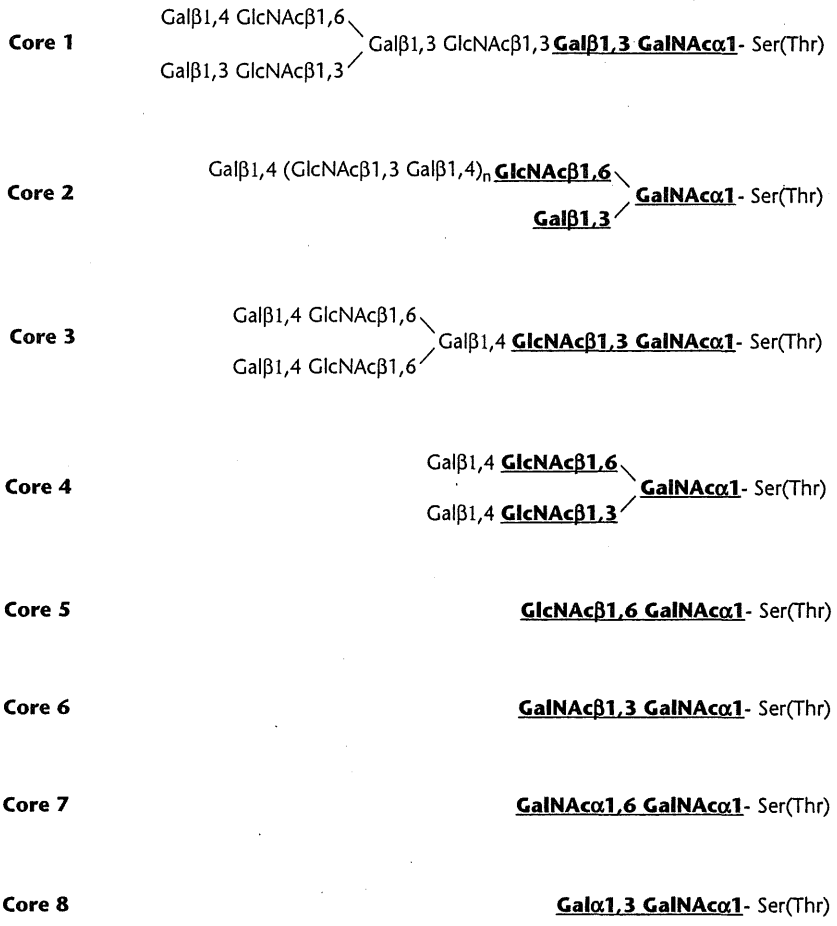

Figure 2. Common core structures of *O*-glycans (bold and underlined). Sugar abbreviations as in *Appendix 1*.

Glycan Heterogeneity

Not only the type of glycosylation (*N*- or *O*-linked), site occupancy, and the site of glycosylation can vary from glycoprotein to glycoprotein, but also the actual oligosaccharide structures (branching and linkages) can differ, even on the same site. This structural variation arises because glycosylation is a process that is not driven by a template. The glycosylation pattern at a given site depends on many factors, including cell-specific and growth-dependent availability of glycosyltransferases and *exo*-glycosidases found in the Golgi bodies and ER. Heterogeneity leads to different physical and biochemical properties and, therefore, also to functional diversity.

Host-Cell Expression Systems and Glycosylation

BACTERIA

Although both *O*- and *N*-glycosylation have been shown to occur in a variety of prokaryotes, *Escherichia coli*, the bacterium of choice for many therapeutic products, does not produce glycosylated proteins.

YEAST

Yeast produces both *N*-glycosylated and *O*-glycosylated proteins. In yeast hypermannosylation with the *N*-glycan chain that contains more than 100 mannose residues can occur, but sialylation does not occur unless the organism is genetically modified. The development of recombinant strains of *Pichia pastoris* that contain inserted heterologous genes for various glycosylation

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enzymes has allowed the humanization of *N*-glycosylation pathways in this yeast. *O*-Glycosylation in yeast is also significantly different from that in mammalian cells. In contrast to mammalian cells, serine or threonine *O*-glycosylation is linked via mannose and often consists of linear chains of as many as six mannose residues.

INSECT CELLS

N-glycan chains of insect cells usually are of the high-mannose, trimannose or paucimannose, and truncated complex types (see *Appendix 1* for definitions). Insect cells also produce glycoproteins bearing the Fucα1,3 residue linked to the proximal GlcNAc residue in the core chitobiose. This core fucose residue is a potent immunogen and allergen. *O*-Glycosylation in insect cells has not been well-studied, and although *O*-linked GalNAc–Ser(Thr) residues have been found, very few are processed further beyond the Galβ1,3GalNAc–Ser(Thr) sequence. Sialic acid residues have not been found on proteins produced in insect cells.

PLANTS AND PLANT CELLS

Plant *N*-glycans contain mainly oligosaccharides of the oligomannose type, but also present are hybrid and truncated complex types of structures, with or without Xylβ1,2 attached to the β-linked mannose residue of the trimannosyl core and Fucα1,3 attached to the proximal GlcNAc residue of the core chitobiose. Both the Fuc and Xyl residues are immunogenic and have been shown to be part of the glyco-epitopes of several plant allergens. *O*-Glycosylation in plants has not been well studied but is known to consist predominantly of the addition of arabinogalactan chains attached to hydroxyproline, threonine, and serine residues that are located in the plant cell wall or on the outer surface of the plasma cell membrane. These glycans are immunogenic.

ANIMAL CELLS

The majority of glycosylated therapeutic proteins are produced in continuous animal cell lines. Chinese hamster ovary (CHO), baby hamster kidney (BHK), human embryonic kidney (HEK), and mouse myeloma (SP2/0 or NS0) cells have all been employed. These animal cells generally produce proteins with humanlike glycosylation. Although there are several differences in glycosylation between rodent and human cells, such as the presence of *N*-glycolylneuraminic acid not found in humans, CHO cells have become a workhorse of the biotechnology industry.

GLYCAN ANALYSIS FOR GLYCOSYLATED BIOLOGICAL DRUGS

Glycosylation of proteins may affect biological activity, either directly or indirectly, and variability in glycosylation arises not only from cellular diversity but also from the manufacturing process. The glycosylation pattern thus may be important as a part of characterization studies in assuring process consistency and may be also be important in ensuring the consistent quality of a biological drug product after market access. Appropriately characterized reference materials are needed in order to support biological and physicochemical testing of production batches to ensure batch-to-batch consistency. Glycosylation analysis may be appropriate for the following:

1. Characterizing the structure and stability of novel products and their stability to processing steps and storage;
2. Batch release testing and in process control testing; and
3. Assessing comparability between products (e.g., when one or more process changes have been made).

An understanding of the relationship between glycan structure and biological function underpins decisions about the information required at each development stage. For biological/biotechnological drug substances, the characterization criteria and specifications for batch release are generally set forth in the guidelines ICH Q6B, *Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*;[1] and ICH Q5E, *Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process*.[2] Numerous approaches and methodologies are applied for glycan mapping. This variety is a consequence of the diversity and complexity of glycan structures and the available technology and detection systems.

Because of the diversity and complexity of glycan structures and the increasing availability and improvement of various detection systems and technology, analytical methods are wide ranging. Different methods that support step-by-step procedures depend on the glycoproteins, the availability of equipment, the expertise of individual scientists and groups, and the information required. The two most studied types of protein glycosylation that affect bioactivity are *N*- and *O*-glycosylation.

Glycan analysis can serve in different applications; the most important are general product characterization, process validation, comparability evaluation, stability testing, monitoring manufacturing process consistency, and release testing. The selection of the analytical techniques and their applications in product development and routine manufacturing depend on many factors, such as the complexity of the glycoprotein, the understanding of the relationships between glycosylation and safety and efficacy, and the overall design of the strategy for manufacturing process control. For example, even when the biological relevance of glycosylation is not certain, control of glycosylation could be considered as a measure of manufacturing consistency.

The *Figure 3A* flow chart assists in the choice of applications for glycan analysis, and *Figure 3B* provides an overview of available analytical techniques and equipment employed.

[1] http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html; accessed 12/15/2011.
[2] http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html; accessed 12/15/2011.

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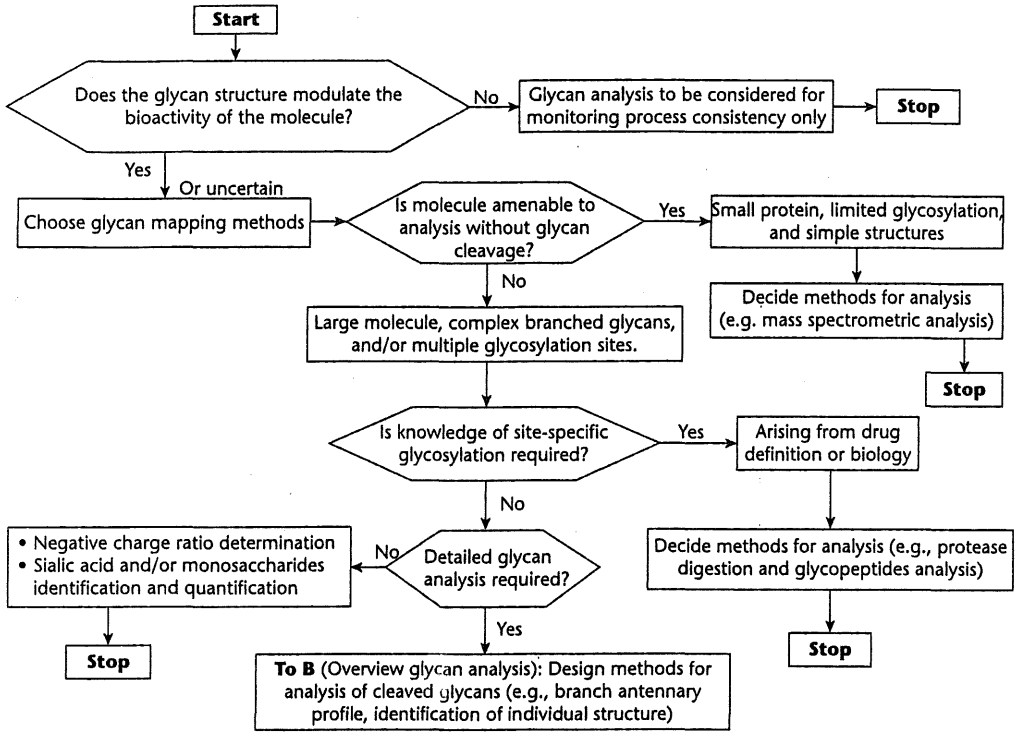

Figure 3A. Flow diagram assisting in the choice of options for glycan analysis.

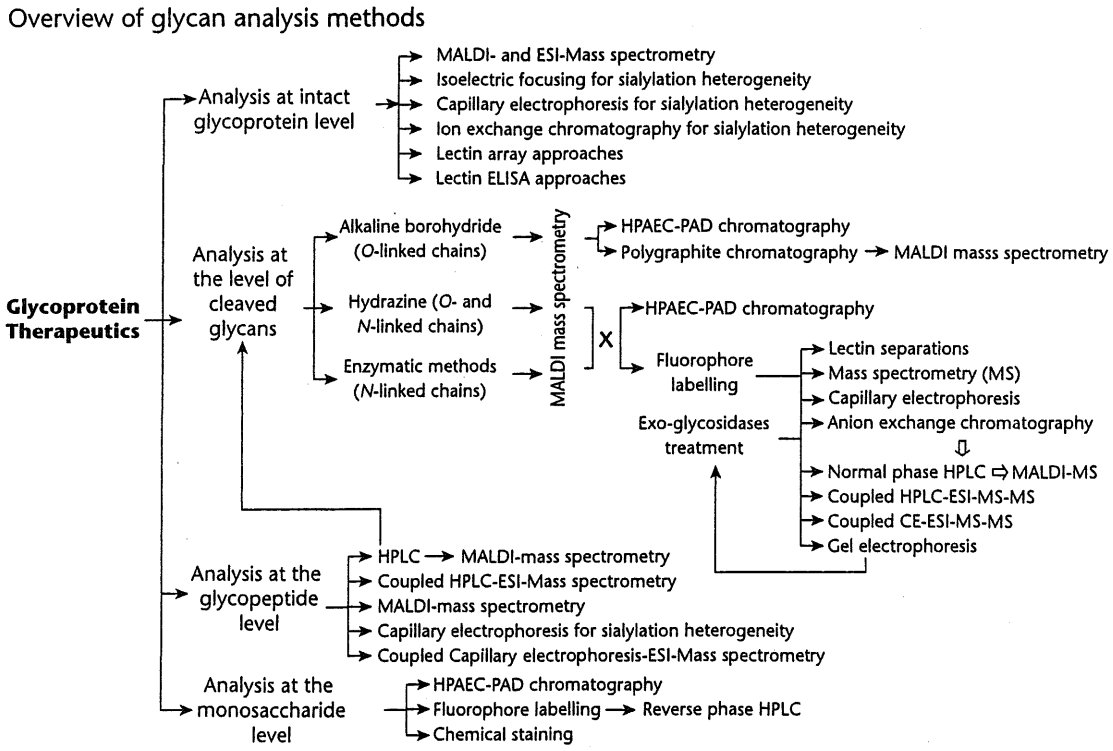

Figure 3B. Overview of glycan analysis techniques and equipment employed.

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CHOICE OF GLYCAN ANALYSIS FOR CHARACTERIZATION AND SPECIFICATION OF GLYCOSYLATED BIOLOGICAL DRUGS

Analysis of Intact Glycoprotein

The most direct mode of analysis is direct study of the intact molecule. This mode provides information about the glycosylation profile of the glycoprotein. However, this approach provides limited information when the molecule is large and contains multiple glycosylation sites. One of the most important glycosylation factors defining biological activity is the degree of sialylation, which often determines the half-life of glycoproteins in circulation. This makes ionic-charge–based electrophoresis and ion-exchange chromatography obvious choices of technique. Nearly all types of gel electrophoresis have been used to probe protein glycosylation, including polyacrylamide gel electrophoresis (PAGE), (see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩) and isoelectric focusing (IEF), (see *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩). Similarly, capillary electrophoresis (CE), (see *Capillary Electrophoresis* ⟨1053⟩) has also been found suitable. Strong anion-exchange chromatography has been used for the same purpose, but the resolution is often inferior to that of IEF and CE. Direct mass spectrometry (MS) is another option for the analysis of posttranslational modification. Along with ongoing improvements in the resolution of MS, more and more complex glycoproteins become accessible for direct characterization by this method.

Analysis of Glycopeptides

Analysis of glycopeptides provides information about site-specific glycosylation properties, the degree of occupancy, and oligosaccharide structures. Site-specific glycosylation can be affected by cell culture process conditions. Therefore, if a known glycosylation site is critical, manufacturers must monitor site-specific glycan structures. The typical approach is first to generate glycopeptides by protease digestion and to separate them by, for example, RP (reversed-phase) HPLC (see *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩). Subsequently the separated glycopeptides can be further characterized individually by, e.g., direct analysis using MS, or deglycosylation and subsequent glycan profiling, as described below in the section *Profiling of Cleaved Oligosaccharides*.

Direct identification of the mixture of glycopeptides and nonglycosylated peptides by MS is limited by masking effects (ion suppression) of peptide signals on glycopeptide signals. One approach to overcoming this effect is to separate peptides and glycopeptides before analysis by MS, e.g., by offline coupling (matrix-assisted laser desorption ionization [MALDI]) or online coupling (electrospray ionization [ESI]). MS analysis of glycopeptides plays an important role in the characterization of *O*-glycans because these glycans are not always released quantitatively and because, as a result of their smaller size, they are more amenable to characterization by MS as glycopeptides.

The use of CE for high-resolution separation may also be appropriate, especially for the analysis of sialylation.

Profiling of Cleaved Oligosaccharides

Profiling of total glycans cleaved from glycoprotein is the most common approach for the characterization of glycoproteins. It provides a way to obtain information about the various populations of glycans present on the protein. The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatization/labeling may be needed to allow the detection of the glycans. Many protocols are available, and most of the steps in the analysis are well established. The possible drawback with such flexibility is the lack of consensus about which methods to choose under which circumstances; because of the variety of analytical techniques, comparison of results obtained by different platforms may not always be possible. So far, the majority of the work has been done on *N*-glycosylation, because of the following factors:

1. *N*-glycans usually are more clinically relevant in biologicals than *O*-glycans; or
2. The release of *N*-glycans, either by chemical means (hydrazine) or by enzymes (endoglycosidases and peptide *N*-glycosidase [PNGase] F), is more straightforward than is the release of *O*-glycans.

DEGLYCOSYLATION

The approach used for the release of glycans depends on the glycoprotein under test. The cleavage agent is chosen according to the type of cleavage needed and the level of information required. Enzymatic or chemical cleavage may be used. *Table 1* gives a nonexhaustive list of enzymatic cleavage agents and their specificity. Digestion efficiency generally depends on the accessibility of the glycans on the protein, and hence the protein should be denatured to maximize glycosylation site exposure unless analysts want to distinguish between surface and buried glycans. Chemical cleavage agents can also be used, e.g., hydrazine or alkaline borohydride for β-elimination of *O*-linked glycans.

Table 1. Examples of Enzymatic Cleavage Agents

Agent	Specificity
***N*-linked glycan release**	
Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52)	Hydrolysis of peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) *N*-acetyl-β-D-glucosaminylamine and a peptide containing an aspartate residue
Peptide *N*-glycosidase F (PNGase F)	Release of *N*-glycan chain but no release of *N*-glycan chain containing (α1,3)-linked core fucose
Peptide *N*-glycosidase A (PNGase A)	Release of *N*-glycan chain containing (α1,3)-linked core fucose

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Table 1. Examples of Enzymatic Cleavage Agents *(continued)*

Agent	Specificity
***N*-linked glycan release**	
Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96)	Endohydrolysis of the *N,N′*-diacetylchitobiosyl unit in high-mannose glycopeptides/glycoproteins containing the –[Man(GlcNAc)$_2$]Asn structure
Endo-β-*N*-acetylglucosaminidase F (endo F)	Release of high-mannose, hybrid, and complex oligosaccharides
Endo-β-*N*-acetylglucosaminidase H (endo H)	Release of high-mannose and hybrid oligosaccharides
***O*-linked glycan release**	
Glycopeptide α-*N*-acetylgalactosaminidase (EC 3.2.1.97)*	Hydrolysis of terminal D-galactosyl-*N*-acetyl-α-D-galactosaminidic residues

* This enzyme has limited usage because of its high substrate specificity.

Chemical or Enzymatic Release of *N*-Glycans—PNGase F (*Flavobacterium meningosepticum*) is the enzyme of choice for the release of *N*-glycans for most glycoproteins except for some insect cell and plant glycoproteins that may contain a Fucα1,3 linked to the chitobiosyl core. *N*-Glycan chains having this structure can be cleaved from the glycopeptide only by the almond enzyme, PNGase A. Chemical release by anhydrous hydrazine is much less common, mainly because of the limited availability of the reagent, which is considered a hazardous chemical. In addition, hydrazinolysis produces de–*N*-acetylated *N*-glycans.
Chemical or Enzymatic Release of *O*-Glycans—Currently only one enzyme, *O*-glycanase from *Diplococcus pneumoniae*, is available to release *O*-glycans, and this enzyme has a limited usage because of its high substrate specificity: it cleaves only Galβ1,3GalNAcα1-Ser/Thr. In addition, no ideal chemical procedure is available; but Ser- and Thr-linked *O*-linked glycan can usually be released by the reductive alkali-catalyzed β-elimination reaction (alkaline borohydride reaction), in which the released glycans are reduced as soon as they are cleaved in order to prevent formation of degradation products due to peeling. However, this reaction is not specific, and in the reaction, approximately 10%–20% of *N*-glycans are generally known to be released as well. The released glycans lack a reducing group used for the attachment of fluorescent labels by reductive amination. Fortunately, with advances in sensitive MS, direct identification of reduced glycans is possible. Relatively good quality reducing *O*-glycans can be obtained by alkali-catalyzed β-elimination using primary amines such as ethylamine and hydrazine. However, both reagents have the potential to produce peeled degradation products. Furthermore, *O*-glycan release by ethylamine is not quantitative. Hydrazine, although it may be better for use than ethylamine, requires strict control of reaction conditions and handling and does not have a commercial source in Europe.
Separation of Cleaved Glycans Without Fluorescent Labeling—*N*-Glycans can also be resolved by HPAEC high-pH anion-exchange chromatography with pulsed amperometric detection (PAD; see *Chromatography* ⟨621⟩), which shows high sensitivity, can also separate some isomers, and affords the ability to directly detect native glycans without labels or tags. However, LC/MS for this separation approach is challenging because this HPAEC system uses high-pH and high-salt mobile phases that interfere with ionization of glycans. In addition, absolute quantification of the glycan is only possible if the individual PAD response factors for the different glycan structures are known, e.g., if an appropriate oligosaccharide reference library is available. Porous graphitic carbon (PGC) chromatography can also be used to separate glycans, and this method adds an orthogonal selectivity compared to other columns. A PGC-electrospray-ionization-MS approach also can be applied for direct glycan analysis.

MALDI/ESI-MS is a powerful method for the analysis of glycan mixtures either in the native or derivatized form. Permethylation of released glycans is a common method for direct analysis using MALDI/ESI-MS especially for sialylated glycans.

LABELING OF GLYCANS TO INCREASE DETECTION SENSITIVITY AND/OR TO MODIFY THEIR PHYSICOCHEMICAL PROPERTIES

Chemical derivatization is the most commonly used method for labeling glycans at their reducing end by reductive amination. One fluorescent label can be attached to each mono- and oligosaccharide, which facilitates determination of molar quantities. *Table 2* illustrates the most common examples of fluorescent labels and their most common uses.

Table 2. Examples of Fluorescent Labels

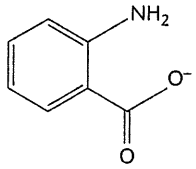
Name	Acronym	Structure	Analytical Technique
2-Aminobenzoic acid	2-AA		HPLC

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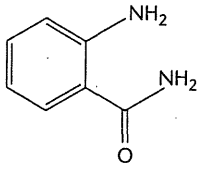
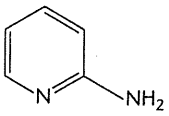
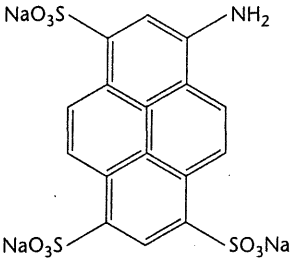
Table 2. Examples of Fluorescent Labels *(continued)*

Name	Acronym	Structure	Analytical Technique
2-Aminobenzamide	2-AB		HPLC MS
2-Aminopyridine	2-AP		HPLC
Trisodium 8-aminopyrene-1,3, 6-trisulfonic salt	APTS		CE

N-GLYCAN PROFILING

Released glycans can be analyzed or profiled by chromatographic, electrophoretic, or MS procedures and, in general, by a combination of these. The choice of method can be grouped according to the nature of the glycans and level of information required. Analysis of glycans provides information about the various populations of glycans present on the protein (high-mannose, hybrid, complex).

Profiling of Glycans by HPLC and/or by Electrophoresis and MS—Profiling of fluorescent-tag–labeled glycans by HPLC has become the most common approach. One label can be attached to every single mono- and oligosaccharide by reductive amination at their reducing end, which facilitates determination of molar quantities. With the appropriate label, glycans can be profiled with high sensitivity using reversed-phase, normal-phase, and anion-exchange HPLC (see *Chromatography* ⟨621⟩). Routinely, analysts use a combination of these methods in order to increase separation resolution and to better differentiate glycan structures. The accuracy of the glycan identification can be validated by means of glycan standards and/or by coupling the HPLC system with MS. Thus, anion-exchange, normal-phase, and reversed-phase HPLC–ESI–MS–MS form powerful combinations; and in-line analysis, if possible, may provide both relative quantitative profiling and information on glycan structure in a single run. Peak identification through retention time is acceptable if their identities have been previously validated by complementary methods and peak homogeneity can be assured.

The degree of sialylation of glycan chains can be a crucial factor for clinical efficacy, because sialylation often defines the half-life of the molecule in vivo. Anion-exchange HPLC is the simplest method for its determination, and glycan structures based on charge can then be identified by MS. Desalting of each fraction is required before MS if the ionization interface is designed for low-salt-containing sample flows only.

High-resolution separation systems such as CE have been used to identify glycan structures without MS when well characterized standards are used for comparison. The development of an online CE–MS system has further increased the power of glycan analysis using this approach.

Structural Identification by Micro-Enzyme Sequencing and Mass Spectrometry—Traditionally, when detailed structural information is required, the analysis is usually performed using micro-enzyme sequencing. This procedure is highly dependent on the specificity and quality of the enzymes used. Recently, tandem MS has been used more regularly to confirm, determine, and sequence known and novel glycan structures; this method is feasible especially when glycans are released from well-known glycoproteins and production sources.

Monosaccharide Analysis

Different quantitative monosaccharide assays are carried out for a number of purposes. In the glycoprotein field, they provide information about the relative amounts of saccharide in a glycoprotein and about the degree of sialylation of a glycoprotein; and by the measurement of monosaccharide composition, they provide some information about the structure of the glycan chains present.

The simplest assays used are colorimetric tests to demonstrate that the product is glycosylated and to quantify the total amount of saccharide present in the product. These have poor specificity between different types of sugar residues.

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Assays of monosaccharide composition are generally simpler to perform than is oligosaccharide profiling, but they provide less information. The most widely used assay is quantification of sialic acid content, because loss of sialylation and exposure of terminal Gal residues may lead to faster clearance of the glycoprotein from the circulation.

The assays can be divided into two types: (1) those that provide compositional information about the intact sample without prior degradation; and (2) others, principally chromatographic, that require hydrolysis of the saccharide chains before analysis and generate quantitative information about several different monosaccharide species simultaneously. In general, the former are colorimetric and the latter are chromatographic. The hydrolysis step is a significant source of assay variability and may require careful optimization for specific samples.

The presence of certain monosaccharides is diagnostic of specific glycan structures. For example, observation of GalNAc is usually a marker for the presence of *O*-linked glycan chains, and fucose denotes the presence of specific types of chains. As a consequence of the limited diversity of monosaccharide residues present in glycoprotein glycans, accurate quantification of Man, Gal, or GlcNAc residues is required in order to distinguish between large numbers of structurally diverse glycans. The monosaccharide *N*-glycolylneuraminic acid (Neu5Gc) is not produced in humans and is generally regarded as an unwelcome and potentially immunogenic component of biopharmaceutical products.

SAMPLE PREPARATION

Glycoprotein samples for monosaccharide analysis should be free of salts, excipients, and other carrier materials (low molecular weight sugars are often used as excipients for biopharmaceuticals). This can be achieved by a number of methods, including the following:

1. Dialysis against water or a volatile buffer, using an appropriate membrane, and lyophilization;
2. HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index, and followed by lyophilization of the sample; or
3. Sample trapping on a conventional RP-SPE cartridge such as a C18 or C8 SPE system, followed by washing away of salts and excipients and elution of the required sample.

QUANTIFICATION

The common method for quantification of neutral sugars in glycoproteins depends on the color generated by heating glycans or glycoproteins in the presence of aqueous phenol in concentrated sulfuric acid. In many cases, the heat required for this reaction is generated by addition of concentrated sulfuric acid to the glycoprotein–phenol mixture in water. Rapid and efficient mixing of the solutions is critical for consistent results. Quantitative results are obtained by the simultaneous analysis of standards to generate a standard curve of absorbance against amount of saccharide and/or against a reference sample of the product under analysis.

HYDROLYSIS PROCEDURES FOR POLYSACCHARIDES AND GLYCOPROTEIN GLYCAN CHAINS

Chromatographic methods for the identification and quantification of monosaccharide components require hydrolysis of the sample before analysis. Appropriate sample preparation is required because excipients or process-related impurities may be saccharides, and residual salts may interfere with the hydrolysis or the subsequent chromatographic separation or with fluorophore labeling. Sialic acid residues can be released either by mild acid hydrolysis or by enzymatic treatment, which leaves other sugar residues attached to the peptide backbone. Quantification of the amount of saccharide present is based on addition of an internal standard before or after hydrolysis. The most commonly used standard for sialic acid analysis by HPAEC is 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), and 2-deoxyglucose is widely used for neutral sugars. Both of these sugars are acid labile and should be added after the hydrolysis step. Accurate quantification depends both on stoichiometric hydrolysis and a lack of degradation of the monosaccharide products during hydrolysis.

DETERMINATION OF TOTAL SIALIC ACIDS

Sialic acids occur in bacterial polysaccharides and glycoproteins generally as *N*-acetyl and *N*-glycolyl derivatives of neuraminic acid (Neu5Ac and Neu5Gc). The sialic acids can be determined together with other monosaccharides by a procedure that includes acid hydrolysis to liberate constituent monosaccharides, followed by HPLC using an appropriate standard mixture. Alternatively, total sialic acid content can be determined by colorimetric procedures without the need for hydrolysis. One method, commonly referred to as the Warren method, is based on the reaction of thiobarbituric acid with the product of periodate oxidation of neuraminic acid released in situ from the glycoprotein. Alternatively, the color can be generated by the reaction of resorcinol with neuraminic acid. For accurate quantification, include a reference standard sample is included in each measurement.

Selective Release of Sialic Acids—Mild acid hydrolysis or enzymatic digestion can be used to selectively release sialic acid from glycoprotein glycan chains for quantification by chromatographic methods and for quantification of unwelcome forms such as Neu5Gc. More aggressive acid conditions are required in order to release neutral and amino sugars before chromatographic analysis. The protocol must be optimized with respect to yield and saccharide degradation for each protein to be analyzed.

Neuraminidase Digestion for the Release of Sialic Acid from Intact Glycoproteins—Several types of neuraminidases have been isolated and studied; the enzyme derived from *Clostridium perfringens* is the one most commonly used for the enzymatic release of sialic acids from glycoproteins. Recombinant enzyme is available from commercial suppliers. Other enzymes with different specificities are available and can be used to distinguish different types of linkages. Hydrolysis conditions should be optimized for each product, because kinetic parameters for different linkages and for Neu5Ac and Neu5Gc may differ. Selective removal of Neu5Acα2→,3-linked and Neu5Acα2→,6- from cleaved glycans is a convenient means of defining linkages. For quantitative analyses, a known quantity of a suitable internal standard, often 2-deoxyglucose, is added after hydrolysis and removal of the acid.

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SEPARATION AND QUANTITATION OF UNLABELED MONOSACCHARIDES

Essentially the only method used for the simultaneous identification and quantification of unlabeled monosaccharide in hydrolysates is HPAEC-PAD. HPAEC-PAD methods are also applicable to oligosaccharide separations, and a single instrumental approach can be used for both applications.

For the general principles and components of chromatography, see *Chromatography* ⟨621⟩. HPAEC-PAD facilitates analysis of monosaccharides and all classes of oligosaccharides without derivatization. Carbohydrates, because they are polyhydric compounds, are weak acids that have pKa values of 12–14, and at high pH even neutral carbohydrates are ionized and can be separated as oxyanions by ion-exchange chromatography. Although separations can be performed on alkali-stable porous polystyrene–divinylbenzene anion exchangers, carbohydrates tend to exhibit broad peaks as a result of mass transfer problems. In microbead pellicular anion-exchange column packings, small functionalized latex beads (<0.1-µm diameter) are attached to larger (<10-µm diameter) uniform nonporous beads. The carbohydrate analyte interacts with the functional groups at the surface of the latex microbeads, eliminating diffusion into and out of pores and the associated peak broadening.

PAD is the method of choice for the detection of carbohydrates in HPAEC because it relies on the high-pH solutions that HPAEC provides by default. Amperometric detection measures the current, or charge, resulting from the oxidation or reduction of analyte molecules at the surface of a working electrode. Electrons are transferred from the electroactive analyte to the electrode during oxidation reactions and in the opposite direction during reduction reactions. This process allows sensitive and highly selective detection of analytes that can be oxidized or reduced, but interfering species that are not electroactive remain undetected. Carbohydrates are easily oxidized at gold and platinum electrodes at high pH, and the current generated is proportional to the carbohydrate concentration.

A typical amperometric detection system contains a working electrode and a reference electrode. Gold electrodes are most common for carbohydrate analysis, but oxidation products poison the electrode surface and inhibit further oxidation. Maintaining a stable, active electrode surface is accomplished by cyclical pulsing between high positive and negative potentials. This timed series of different potentials is referred to as a waveform, and repeated application of a waveform is the basis of pulsed amperometry. Different waveforms are used for different HPAEC-PAD applications and for different working electrodes: disposable gold electrodes require the use of fast, quadruple waveforms, but other gold electrodes allow a wider range of waveforms to be used without damaging the electrode surface. Disposable electrodes and fast waveforms were introduced to minimize the influence of electrode recess on the sensitivity and precision of quantitative monosaccharide applications.

FLUOROPHORE LABELING OF MONOSACCHARIDES BEFORE SEPARATION AND QUANTIFICATION

An alternative approach to the identification and quantification of monosaccharides present in a hydrolysate is to modify the monosaccharides by reductive amination with an easily detected fluorophore label that allows high-sensitivity detection and improves the chromatographic separation of monosaccharides. Essentially standard HPLC equipment can be used and, because the same labeling approaches are applicable to cleaved oligosaccharides, a consistent analytical approach can be applied. Fluorophore labeling has been much less widely used than HPAEC-PAD for monosaccharide identification and quantification. Labeling of sialic acid derivatives is usually undertaken with 1,2-phenylenediamine (or DMB, the 4,5-methylenedioxy derivatives), and the resulting products are separated on a C-18 column and using fluorescence detection.

CONCLUSION

Because of the complexity of glycoprotein glycan structures and their inherent variation during production processes, manufacturers are generally required by means of characterization studies to develop criteria for the control of the glycosylation pattern of a biological drug substance when glycosylation occurs, as well as to develop the level of information required at each stage of production and at batch release. Then analytical procedures can be derived in a manner that provides information relevant to fulfilling quality requirements. In general, a combination of approaches and techniques is needed, and more detailed glycan structural analysis at early drug development stages is required. Validation considerations are central as method development and product knowledge progress.

APPENDIX 1

Abbreviations

Fuc	L-Fucose
Gal	D-Galactose
GalNAc	*N*-Acetyl-D-galactosamine
Glc	D-Glucose
GlcNAc	*N*-Acetyl-D-glucosamine
Man	D-Mannose
Neu5Ac	*N*-Acetylneuraminic acid
Neu5Gc	*N*-Glycolylneuraminic acid
Xyl	D-Xylose

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Additional Definitions

High mannose—Glycan chains containing two core GlcNAc residues and between five and nine Man residues, and lacking Gal, GlcNAc, or Neu5Ac residues in the antennae. Such chains are typically found in mammalian glycans.

Hypermannosylation—(i) Addition of Man residues to high mannose chains creating chains with large numbers of Man residues, and (ii) *O*-Man linked glycan chains with multiple Man residues synthesized by yeast.

Paucimannose—Glycan chains containing two core GlcNAc residues between two and four Man residues. Core-linked Fucα1,3 and/or Fucα1,6 residues may be present.

Oligomannose—Used here as a generic term to include high mannose, paucimannose, and *N*-linked hyper-mannosylated chains.

Add the following:

▲⟨1085⟩ GUIDELINES ON THE ENDOTOXINS TEST

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INTRODUCTION

The first version of *Bacterial Endotoxins Test* ⟨85⟩ appeared in *USP 20–NF 15* (1980). The chapter was subsequently harmonized with the *Japanese* and *European Pharmacopeias*, and the first harmonized chapter appeared in *USP 25–NF 20* (2002). Since its first publication, the content has changed very little, but years of experience, increasing knowledge, and more complex parenteral formulations suggest that the basic methodologies described herein could benefit from additional supporting information. The purpose of this information chapter is to provide additional background information and guidance for the performance and proper application of the compendial bacterial endotoxins tests.

BACKGROUND: PYROGENS AND ENDOTOXINS

Pyrogens, or fever-causing agents, are possible contaminants in parenteral products. Many substances can cause fevers when injected, infused, implanted, or when they come in contact with the bloodstream or cerebrospinal fluid of mammals. Although some biologics, vaccines, and cell and gene therapies may, by their nature, elicit pyrogenic responses in patients, the

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predominant and most potent pyrogenic contaminants in the manufacturing of parenteral drugs and medical devices are bacterial endotoxins, which are components of the cell walls of Gram-negative bacteria (GNB).

Endotoxins are integral with the outer cell membrane of GNB. If GNB cannot grow, endotoxins cannot be generated. However, endotoxins may remain active in cell wall fragments after cells die, so a material may be sterile but may still contain quantifiable levels of endotoxins activity. Bacterial endotoxins, when present in parenteral products (including biological products) or medical devices, indicate that the growth of GNB occurred at some point during manufacture of the product. Endotoxins can be introduced into the process stream by pharmaceutical ingredients including water, raw materials (particularly from natural sources), the active pharmaceutical ingredients (API), drug product formulation excipients, primary (product contact) packaging components improperly cleaned or stored manufacturing equipment, and/or ineffective microbial contamination control practices.

ENDOTOXINS

The terms "bacterial endotoxins" and "endotoxins" refer to a complex component of the outer cell membrane of GNB. Although still an active area of research, natural contaminants in sterile parenteral drug products or medical devices are thought to include outer membrane vesicles (OMV), which are mini-spheres of outer membrane material that are released from actively proliferating cells as part of the normal bacterial growth cycle (*1–6*) as well as outer membrane fragments that come from disrupted or dead GNB cells. The biologically active lipopolysaccharide (LPS) is embedded in or associated with other Gram-negative outer membrane components, including various proteins, phospholipids, and lipoproteins (*1,3,6*). The hydrophobic lipid A portion of the LPS molecule is embedded in the membrane and the hydrophilic polysaccharide portion is exposed to the external environment surrounding the cells. Some current research suggests that it is doubtful that pure LPS is released as part of the normal growth cycle of GNB (*3*).

The current USP Endotoxin Reference Standard (RSE) and commercially prepared control standard endotoxins (CSE) are extracted from the GNB cell membrane, generally by the Westphal hot phenol method, and are purified to remove any surrounding cell membrane components (*7,8*). Additionally, these purified preparations are often further formulated with stabilizing agents (*8*). As such, LPS prepared using Westphal, or other extraction methods, cannot contaminate pharmaceutical products because they do not exist in this form in nature. Endotoxin standards, attributed to differences in source and manner of preparation, could "react differently from native sources of endotoxins" (*9*). It is possible that standards extracted by the Westphal method or other denaturing methods and then formulated may not always be representative surrogates for modeling the behavior of natural endotoxins in some pharmaceutical, biopharma, or medical device extraction experiments (*10,11*).

Although general test methods in the compendial chapters numbered below ⟨1000⟩ are considered to be validated, all laboratories, including contract testing laboratories, must demonstrate that the chosen BET methodology is suitable for a specific product or material tested according to *Bacterial Endotoxins Test* ⟨85⟩, *Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors* or *Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors* (also known as inhibition/enhancement testing). This suitability testing will require the following prerequisites:

- Calculation or assignment of an endotoxins specification or limit for the material under test, and calculation of the maximum valid dilution (MVD). See the following sections: *Method Suitability, Calculating Endotoxin Limits for Drug and Biological Products, Calculating Endotoxin Limits for Medical Devices, Calculating Endotoxin Limits for Combination Products,* and *Calculating Endotoxin Limits for Active Substances and Excipients* below.
- Demonstration that the positive product control (PPC) can be recovered at a dilution of material that does not exceed the MVD. See *Bacterial Endotoxins Test* ⟨85⟩, *Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors* or *Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors.*

Once assay suitability has been demonstrated, the laboratory may perform routine testing according to the calculations and sample preparation conditions that were described in the suitability study. Changes to those conditions (e.g., the lysate and/or endotoxins source, product component(s), formulation, or changes in manufacturing) are subject to change control and may require that the laboratory repeat the suitability study.

PREPARATORY REQUIREMENTS

RSE: CSE Calibration

It is well known that purified LPS from different genera/species/strains of GNB, when experimentally administered to rabbits on a uniform weight or mass basis, can produce significantly different pyrogenic reactions (*12,13*). However, by relating these effects to the administered activity—meaning their ability to elicit a fever in the rabbit (*Pyrogen Test* ⟨151⟩) or initiate an LAL reaction (⟨85⟩)—rather than weight or mass, the structural variability of LPS molecules can be normalized to a defined unit of activity called the endotoxin unit (EU). Two notes apply here:

- One USP EU is equivalent to one International Unit (IU) as indicated in ⟨85⟩.
- Lysate reagents currently described in ⟨85⟩ are licensed in the United States by the FDA and have a sensitivity associated with them; however, the FDA does not license CSE preparations.

The RSE is the purified LPS primary standard that is formulated from a common bulk preparation of *E. coli* 0113:H10:K LPS that is shared among the World Health Organization (WHO), *European Pharmacopoeia* (*Ph.Eur.*), *Japanese Pharmacopoeia* (*JP*), and *U.S. Pharmacopeia* (*USP*). The RSE was originally developed by the FDA in the 1970s to calibrate lysate reagents from multiple manufacturers, and it remains the primary standard for lysate calibration, calibration of secondary standards (e.g., CSE), and the generation of assay parameters such as standard curves and PPC assay controls.

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Although RSE is available from USP for routine use, most BET assays are performed using a CSE, which is a secondary calibration analyte that may be included in LAL test kits purchased from reagent manufacturers. Many CSEs are provided as lyophilized preparations of a purified LPS that was filled and packaged by weight, not by activity. The purpose of the standardization study is to determine the specific activity, or potency, of the CSE in EU/unit of weight of the material against the RSE primary standard.

All the reagents used in BET assays are biological in nature and, therefore, can exhibit some variability in sensitivity and potency, making calibration against the RSE an important task. Calibration against RSE is necessary for each unique combination of lysate lot and CSE lot. If a kit is purchased from a reagent vendor, the vendor will conduct the lot-specific standardization and provide a lot-specific certificate of analysis (CoA) in the kit, which should be retained for reference in the laboratory. However, there may be circumstances when a laboratory might choose to purchase CSE from a third party or to calibrate a liquid endotoxin preparation, which requires that the laboratory conduct its own calibration study.

CSE Calibration/Potency Determination Using the Gel-Clot Method

CSE potency determination in the gel-clot test is accomplished by comparing the geometric mean (GM) endpoints of separate dilution series of RSE and CSE made in Water for BET [see *Bacterial Endotoxins Test* ⟨85⟩, *Reagents and Test Solutions, Water for Bacterial Endotoxins Test (BET)*] and tested in quadruplicate. The endpoint is defined as the last tube in a two-fold series of RSE or CSE dilutions showing a positive reaction (gel). To have an endpoint, the last positive tube must be followed in the series by at least one negative dilution. The GM is calculated as follows:

$$\text{Geometric mean} = \text{antilog}\left\{\frac{\Sigma\log_{10}\text{endpoints}}{\text{number of replicates}}\right\}$$

The RSE is labeled in units of activity (EU/vial), and the units for the GM endpoint for the RSE are expressed as EU/mL. Because the CSE is filled by weight, the units for the GM endpoint for the CSE are expressed as ng/mL. The potency of the CSE in EU/ng is calculated as follows:

$$\text{Potency of the CSE (EU/ng)} = \frac{\text{GM endpoint of the RSE series in EU/mL}}{\text{GM endpoint of the CSE series in ng/mL}}$$

For example, to calibrate a new lot of CSE for use with a gel-clot lysate lot with a labeled sensitivity of 0.125 EU/mL, dilute the RSE in EU/mL to bracket the label claim of the lysate reagent. For this example, the GM of the RSE confirms the label claim of 0.125 EU/mL. Dilute the CSE in ng/mL so that an endpoint will be reached. For this example, the endpoint of the CSE series is 0.00625 ng/mL. The potency of the CSE for the particular lysate lot is calculated as follows:

$$\frac{\text{GM of the RSE}}{\text{GM of the CSE}} = \frac{0.125\ \text{EU/mL}}{0.00625\ \text{ng/mL}} = 20\ \text{EU/ng of CSE}$$

For this lot of lysate, the potency of the new CSE lot is 20 EU/ng. If the lab wants to use this CSE lot with a different lysate lot, the calibration study must be repeated because potency determination references only a specific combination of lysate lot and CSE lot.

CSE Calibration/Potency Determination Using Quantitative Kinetic and Endpoint Assays

Determining the potency of CSE in a quantitative assay is performed much like a standard assay for an unknown. Onset/reaction times from dilutions of the CSE diluted in ng/mL are interpolated from an RSE standard curve to yield the activity of the CSE dilution in EU/mL. Since the concentrations in ng/mL of these dilutions are known, calculation of EU/ng is a simple mathematical function. For any given concentration of CSE, calculate as follows:

$$\text{EU/ng} = (\text{EU/mL}) \div (\text{ng/mL})$$

For example, to calibrate a new CSE lot for use with a chromogenic assay, the first step is to prepare a standard curve using RSE in Water for BET. The standard curve range is left to the discretion of the analyst, but appropriate choices would be either the same range as is routinely used in the laboratory or the maximum range suggested by the reagent manufacturer. Next, the analyst dilutes the CSE to a known concentration in ng/mL and tests the dilutions as unknowns (see *Table 1*). Points to consider:

1. Because of linearity requirements for a standard curve, data used in the calculation of potency may not be extrapolated beyond the range of the curve, but rather must fall within the range of the referenced standard curve
2. Standard curves should not exceed the maximum ranges that are suggested by the reagent manufacturer
3. For CSE calibration, it is recommended that replicate determinations for any given dilution be considered individually (not averaged) when calculating the correlation coefficient to assess variability when calibrating the CSE

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Table 1. Example of Calibration of a CSE Analyte Against RSE in a Quantitative Assay

Example concentration (ng/mL)	Result (EU/mL)	Result (EU/ng)
1	15	15
0.5	6	12
0.25	3	12
0.125	1.8	14
—	—	Average: 13.25

In this case, the potency of the new CSE lot with kinetic chromogenic reagent is the mean of the four separate determinations. That calculation is 13.25 EU/ng, which is properly rounded down to 13 EU/ng. If the lab wants to use this CSE lot with a different lot of lysate—be it kinetic, endpoint, or gel clot—the calibration must be repeated because potency determination references a specific combination of lysate lot and CSE lot.

Activity Determination for a Liquid CSE

A liquid CSE such as a native endotoxin preparation does not have potency unless the actual weight of the source material is known (*Endotoxin Indicators for Depyrogenation* ⟨1228.5⟩). Typically, liquid CSE are described in terms of a concentration of activity expressed in EU/mL. The activity of a liquid CSE in EU/mL is determined by GM endpoint determination for replicate tubes in gel clot or by treating the solution as an unknown against an RSE standard curve for quantitative assays.

Screening and Qualification of Consumables: Compendial Requirement

The harmonized compendial chapter on the BET requires that the laboratory screen consumable plastics for test interferences. From ⟨85⟩, *Apparatus*:

> If employing plastic apparatus, such as microplates and pipet tips for automatic pipettes, use apparatus that is shown to be free of detectable endotoxins and does not interfere in the test. [NOTE—In this chapter, the term "tube" includes any other receptacle such as a microtiter well.]

Plastics used in the performance of the test are molded, meaning that plastic pellets have been heated to a very high temperature to produce molten plastic in preparation for the molding process. This high temperature will destroy bacterial endotoxins, so molded plastics are free of detectable endotoxins when they are released from the molds. However, depending on the subsequent handling, recontamination is possible. Control measures should be in place to ensure appropriate storage conditions and avoid contact with potentially contaminating substances. However, unless depyrogenated plastics are stored under damp conditions or encounter substances during handling in which GNB could proliferate, the probability of recontamination is remote.

If a laboratory accepts a vendor CoA for the endotoxin content of a disposable component, there should be an understanding of the methods used to determine the reported test result. Two examples of CoAs are provided below:

- If a shipment of dilution tubes is received from a vendor and is labeled "non-pyrogenic", what does this really mean? Was the lot tested for pyrogens using a rabbit pyrogen test or a validated monocyte activation test? Was it tested using a standard ⟨85⟩, and if so, at what level of endotoxin does the vendor consider the material to be "non-pyrogenic"? It is important to ask the vendor how the material was prepared and tested.
- If a shipment of 10-mL tubes is received from a vendor with the label of "<0.5 EU/mL", what does that mean? With what volume was the tube extracted (1, 5, or 10 mL)? A 1-mL extraction would mean <0.5 EU/tube, whereas a 5-mL extraction would mean <2.5 EU/tube and a 10-mL extraction would mean <5 EU/tube. The laboratory should ask the vendor about the method of endotoxin extraction from the tube as well as the BET test conditions that were used to generate the result.

A default method to confirm the CoA result is to treat the plastic disposable as if it were a medical device and proceed according to the methodology provided in *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩. However, while ⟨161⟩ defines the endotoxin limit for medical devices as <20 EU/device, a laboratory may want to consider redefining the limits for disposables used in the test to be less than the value of the most sensitive test used in the laboratory. For example, if the laboratory is using sterile polystyrene tubes for sample dilution, and the most sensitive test is a kinetic chromogenic test with a $\lambda = 0.05$ EU/mL, then consider setting a limit low enough to prevent interference across all test methods.

Likewise, some materials could contain extractable or leachable substances that could inhibit the LAL-bacterial endotoxins assay. The PPC will indicate whether the normal, routine use of the plastic results in any leached inhibitory substance. If a laboratory is considering use of disposable plastic containers such as sterile polystyrene for long-term storage of materials that ultimately will be tested, it is suggested that they do testing to confirm that there are no inhibitory or enhancing factors that could affect the accuracy of the test.

Analyst Qualification

General laboratory training for analysts is good laboratory practice, as well as a current good manufacturing practice (cGMP) requirements (*Microbiological Best Laboratory Practices* ⟨1117⟩). Typically, training for performing any BET involves

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demonstration of acceptable proficiency for both sample preparation and assay method(s). It is suggested that training be divided into two parts:

1. Classroom training delivered by a subject matter expert (SME) develops an understanding of the principles and limitations of the test methods as well as the effects of the analyst's technique on the test result. Differences between analysts in the accuracy and precision of executing basic laboratory tasks such as pipetting, weighing raw materials, and making dilutions may introduce bias. Due to the inherent variability of the reagents (lysate and CSE) routinely used in the laboratory, poor or inconsistent techniques may significantly affect the accuracy of test results. Analysts must be retrained if a new test method is introduced into the laboratory (e.g., a change in laboratory procedures from gel clot to a kinetic assay).
2. Training effectiveness should be confirmed by the demonstration of analyst competency in performing the test. Competency or proficiency training may be divided into two parts:
   A. For compendial assays where confirmation of assay sensitivity is required, it is recommended one part of performance training follow the provision in *Bacterial Endotoxins Test* ⟨85⟩, *Gel-Clot Technique, Preparatory Testing* or *Photometric Quantitative Techniques, Preparatory Testing*. For the gel-clot method, this is the test for confirmation of labeled lysate sensitivity, and for quantitative tests, this requires the construction of a linear standard curve. For training on quantitative tests, it is recommended that replicate determinations for any given dilution be considered individually (not averaged) when calculating the correlation coefficient to assess the variability in the proficiency study. Both of these proficiency assessments require analysts to prepare and dilute RSE or CSE standards. If the laboratory uses a cartridge system with an on-board archived standard curve, it is recommended that the laboratory devise another method of ensuring that the analyst's technique in sample preparation and dilution is sound. This proficiency training should be described, justified, and included in the firm's standard operating procedures to ensure consistency across all analysts. Although there are many ways to determine proficiency, one possibility is to ask the analyst to prepare a sample with a known and confirmed level of endotoxin activity and ultimately compare the result to the known value. If the calculated values are incorrect, this may raise a concern about the analyst's technique.
   B. The second part of the proficiency training should be an "on the job" training where analysts are required to demonstrate their ability to calculate endotoxin limits, prepare samples according to the results of suitability testing, and appropriately execute positive and negative controls in accordance with product-specific instructions and ⟨85⟩.

The following should be stressed during training:

- Appropriate laboratory aseptic technique is important so the analyst does not contaminate samples, diluents, or accessories used to perform the test.
- Use of a vortex mixer or another validated method (e.g., sonication for sample preparation) is important to optimize the distribution of endotoxins in samples and the aggregation state of the purified standards in the standard series. Because the formulations of the CSEs provided in LAL test kits are proprietary, it is highly recommended that analysts follow the manufacturers' instructions for vortexing time, both for reconstitution of the vial of LPS and in between dilutions. However, vortexing of lysate is not recommended as it may result in bubbles in the reagent.
- If reagents are saved, ensure that the "open" date and "expiration" date are clearly marked on the primary containers and that any holding of unused reagents follows manufacturers' instructions.
- Do not store RSE or CSE dilutions without a validation study that includes vessel type and materials of manufacture, concentrations of RSE or CSE that are to be held, hold temperature, and volume of the dilutions to be held.
- When reading gel-clot results, pick tubes up one at a time and invert 180°. Picking up more than one tube could jostle the contents. Once a gel is broken, it will not re-form, and the result may be a false-negative.
- When inoculating a microplate, tube, or cartridge care should be taken to avoid the formation of bubbles, because they will impact the accuracy of the test result.
- When using heating equipment (e.g., bead baths, water baths, plate readers), be certain that the equipment is qualified.
- If using a water bath for gel-clot incubation, change the water frequently. The recommended frequency is at least once a week.
- Ensure that all mechanical pipettors are calibrated, and use them only within the calibrated range.
- When possible, use larger volumes (milliliters) for dilution rather than smaller volumes (microliters), as smaller volumes increase variability.
- Standard curves for photometric tests are constructed based on the $\log_{10}$ of the measured onset or reaction times as a function of $\log_{10}$ of the endotoxin concentration. Pay attention to the onset times of the standards to ensure that they are consistent from run to run, analyst to analyst, and day to day for any given combination of CSE lot and lysate lot. See *Routine Testing, Standard Curve Control*.
- If using a monocyte activation test, be sure to include at least one non-endotoxin control.

An analyst requires additional training if any of the following is noted by a supervisor:

- Failure to meet the requirements of the initial performance training.
- Frequent inability to meet system suitability parameters, yielding invalid test results (e.g., confirmation of label claim for gel clot, demonstration of linearity for quantitative assays, inability to ensure that negative controls are nonreactive). Note that the inability to recover the PPC within the required range may signal an issue with analyst technique or a change in the product's manufacturing or formulation that changes the product's interference profile.
- Erratic results for slope and *y*-intercept for quantitative assays. See *Routine Testing, Standard Curve Control*.
- Adverse trends for out-of-specification (OOS) or out-of-trend (OOT) test results.

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Equipment and Instrumentation Calibration and Qualification

All instrumentation and equipment used in the performance of an LAL test, including (but not limited to) mechanical pipettors, water baths, heat blocks, and incubating plate readers, should be qualified using proper scientific standards and according to approved protocols and maintenance schedules. Where appropriate, user requirement specifications (URS), as well as IQ/OQ/PQ protocols and reports, should be written and ultimately approved by the quality unit. See *Analytical Instrument Qualification* ⟨1058⟩ for additional guidance. All equipment used in performing the BET should be properly calibrated and maintained at frequencies that are in accordance with the equipment manufacturer's recommendations. Because BET incubation temperatures are critical, equipment such as incubating plate readers, heat blocks, and water baths should be evaluated for uniformity of heat distribution.

Incubating plate or tube readers should reference a URS, an installation qualification (IQ), an operational qualification (OQ), and a performance qualification (PQ). Very often the vendors of these instruments will provide the laboratory with IQ/OQ/PQ templates and hands-on assistance that are very useful for the execution of these studies.

If a laboratory or production dry heat oven is used to depyrogenate glassware or other heat-stable items used in the performance of any of the BET assays, it must be validated to ensure appropriate time/temperature exposure and load pattern (see *Dry Heat Depyrogenation* ⟨1228.1⟩ for additional guidance).

Computer software must be compliant with all federal regulations and standards (21 CFR part 11 in the United States). It must allow for individual user passwords and audit trails. Quality control should understand how the vendors of the BET software have programmed their calculations. For example, if the instrument reports out an averaged result for the assay of three individual samples tested in duplicate, did the software program average the onset times to get the result or did it average the replicate results?

Laboratory Environmental Conditions

The BET can be performed in most modern laboratories under controlled conditions. Appropriate aseptic technique is important when preparing and diluting standards and handling samples. Gowning practice outside of normal laboratory personal protective equipment (PPE) requirements is not a concern unless the product under test demands specific analyst safety considerations due to toxicity or infectiousness. Gloves should be talc-free, as the talc may contain significant levels of endotoxins. Plate readers, water baths, and dry heat blocks used for sample incubation should be on a laboratory bench away from heating, ventilation, and air conditioning (HVAC) ducts, significant vibration, and laboratory traffic that could affect the test results. Sample hold times and conditions should be determined and subsequently documented, if necessary, to ensure that accurate test results can be generated in the qualified time. For example, if the laboratory receives a *Water for Injection* (WFI) or in-process sample, must it be refrigerated or can it remain at room temperature, and for how long? Prior to testing, it is recommended that the primary sample container(s) be adequately mixed before removing the test aliquot(s) for either direct testing or subsequent dilution.

METHOD SUITABILITY

Calculating Endotoxin Limits for Drug and Biological Products

An endotoxin limit specification for a compendial article is the allowable amount of endotoxin activity that can be safely contained in a parenteral product, according to current understanding and experimental evidence (*5*). The endotoxin limit calculation for any drug product is dependent on three variables: 1) the route of administration, which largely defines *K*, the numerator in the endotoxin limit formula, 2) the dose of the product per kilogram of body weight, and 3) the duration (time) of administration. This information can be found in the package insert for an approved drug product or can be obtained from the product development group for a product that is either still in development or in early clinical trials.

An endotoxin limit specification is calculated for each drug product formulation and set of administration conditions as follows:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of 5 EU/kg for most routes of administration or 0.2 EU/kg for intrathecally administered drugs (see *Table 2*)

M = maximum recommended dose of product per kilogram of body weight of the patient. This dose relates to the concentration of active ingredient (potency of the active ingredient) in the finished product formulation. If a product is infused or injected into a patient at frequent intervals over an extended time, then *M* is based on the maximum total dose administered in a 1-hour period. If the pediatric dose per kilogram per hour is higher than the adult dose, the pediatric dose must be used for the calculation.

When calculating the endotoxin limit specification, body weight is defined according to the intended patient population, which can differ in terms of geographical regions or patient populations. For example, the average adult in the United States is assumed to weigh 70 kg, whereas the average adult in Japan is assumed to weigh 60 kg (*14*). Pediatric patients could be 30 kg or below. The average weights for children can be found on the Centers for Disease Control and Prevention clinical growth charts page (see www.cdc.gov/growthcharts/clinical_charts.htm). The body weight factor selected for pediatric and other special category patients should consider the worst case, i.e., lowest body weight in targeted patient populations that

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can receive the greatest recommended dose. There is also a special consideration with respect to body weights for veterinary products. Veterinary drug products may be administered to a variety of different species or subspecies. Generally, the smallest animal will have the greatest dose per kilogram. Reference to the product package insert is highly recommended when establishing veterinary endotoxin limit specifications.

Different routes of administration or types of product, e.g., radiopharmaceuticals or oncology products administered per square meter of body surface, have defined values for *K* in the endotoxin limit calculation as described in ⟨85⟩ and above. Where the product package insert describes multiple patient populations, indications, and routes of administration, it is suggested that the laboratory calculates the limit specification for each administration and chooses the most conservative one as the endotoxin limit specification for the product. A summary is shown in *Table 2*.

Table 2. Defined Values for *K* in Terms of Route of Administration

Route of Administration	*K*	*M*
Intravenous (IV) for parenteral products	5 EU/kg of body weight	Maximum dose per kilogram administered in 1 h
IV for radiopharmaceuticals	175 EU	Volume of the maximum recommended dose
Intrathecal (IT) for parenteral products	0.2 EU/kg of body weight	Maximum dose per kilogram administered in 1 h
IT for radiopharmaceuticals	14 EU	Volume of the maximum recommended dose
Parenterals administered per square meter of body surface	100 EU/m²	Maximum dose per square meter per hour
Injections other than IV (intramuscular, subcutaneous, etc.)	5 EU/kg of body weight	Maximum dose per kilogram administered in 1 h
Intraocular fluids	0.2 EU/mL (*15*)	—
Anterior segment solid devices	0.2 EU/device (*15*)	—
Ophthalmic irrigation products	0.5 EU/mL (⟨771⟩)	—
Injected or implanted ophthalmic drug product	2 EU/dose (⟨771⟩)	—

Some *USP* product monographs have endotoxin specifications defined at a targeted concentration for administered product. However, endotoxin limit specifications should be calculated for all indications in the product's package insert because indications and administrations for the product may be different from the data used to calculate the original *USP* monograph limit. If a firm's most stringent limit is lower than the USP limit, the firm should use its lower calculated endotoxin limit.

It is important that the endotoxin limit specification, as a critical quality attribute for a new product, be calculated early in development and monitored throughout development and early-stage clinical trials. If a dose has not been established, the limit should be calculated based on the worst case (highest) dose that is anticipated for the product with respect to the target patient population and the route of administration. Early phase endotoxin limits may change based on dosing and/or formulation changes prior to commercialization.

Relevance of Limits for Compounded Sterile Preparations

When sterile compounding pharmacies prepare therapies for injection or infusion, care must be taken to avoid the addition of endotoxins to the preparations. The compounding pharmacy should use only product contact materials that they have depyrogenated in house or that they have received as sterile and free of detectable endotoxins (see *Screening and Qualification of Consumables: Compendial Requirement* earlier in the chapter). When diluents or intravenous (IV) solutions are used for preparing a product intended for IV, IM, intraocular, or intrathecal (IT) administration, the diluents should be commercially obtained and should meet the compendial limits, which most commonly are 0.5 EU/mL. If the required diluent is not a *USP* monograph article, it should be manufactured to meet the compendial limit of 0.5 EU/mL. If the required diluent is to be used for IT administration, it is essential that the laboratory ensure that the diluent plus drug product does not exceed the more stringent IT endotoxin limit.

Calculating Endotoxin Limits for Active Substances and Excipients

The control of the levels of endotoxins in excipients and active substances can minimize the risk of finished drug product contamination. Suppliers and drug manufacturers should perform risk assessments of these substances based on raw material origins, production methods, representative sampling and testing, and storage conditions. For example, materials of natural origin such as sugars, heparins, and enzymes may contain significant levels of endotoxins and/or glucans. Suppliers of these materials should be audited or closely evaluated to ensure control of bioburden and endotoxins in their manufacturing operations. If these suppliers provide a CoA regarding the endotoxins or glucan content for individual lots of material, the evaluation should also include an assessment of the validity of test methods and the accuracy of test results and any testing history.

Consideration should be given to using a glucan blocker for products or materials that may contain glucans in addition to endotoxins. Glucan blockers are used to ensure that all tests are specific to endotoxins, preventing a false-positive reaction of the test to glucans. Because products do not have glucan specifications, glucan-blocking reagents are specific to assay methods and lysate formulations and are generally offered by lysate reagent manufacturers. Because glucan-blocking reagents are used

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in different ways, it is recommended that laboratories follow the reagent manufacturers' instructions for the use of these reagents.

If noncompendial materials or articles are tested and released in-house, endotoxin limits should be assigned after a thorough understanding of their potential contribution to the formulation. Working backwards from the calculated drug product specification for endotoxins, limits can be assigned to each individual component in the formulation with the assurance that the drug product limit would not be exceeded if each component were at its limit. If the same component is used in multiple formulations, the lowest limit should be used for the testing and release of that component.

Calculating Endotoxin Limits for Combination Products

A combination product is defined as a product comprised of two or more regulated components (i.e. drug/device, biologic/device, drug/biologic, or drug/device/biologic) that are physically, chemically, or otherwise combined or mixed and produced as a single entity. It also is defined as two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and drug products (*16,17*). Typically, these products can be two or more regulated components presented as a single-entity product (e.g., prefilled syringe), or a kit where two or more regulated products are packaged for use together as a single-entity product (e.g., a lyophilized drug packaged with a diluent and a syringe). Although a manufacturer can propose and justify unique limits for review in regulatory submissions, there are some general points to consider:

- If the combination product is a drug/device combination such as a prefilled syringe, the prefilled syringe may be tested as a filled unit and the endotoxin limit for the drug product prevails. Any endotoxins contributed by the container (device) are assumed to be eluted with the drug product during sample preparation and, therefore, are accounted for in the product's assayable endotoxins.
- If the combination product is two drugs to be administered simultaneously [IV or intramuscular (IM)], then the endotoxin content of the combined dose may not exceed the endotoxin limit for drugs of 5 EU/kg/h for IV or IM administrations or 0.2 EU/kg/h for IT administration.
- If the combination product is a kit containing multiple components that are administered as a single entity (e.g., a lyophilized product/diluent/syringe) the endotoxin content of the combined dose may not exceed the endotoxin limit for drugs of 5 EU/kg/h for IV or IM administrations or 0.2 EU/kg/h for IT administration.

Calculating Endotoxin Limits for Medical Devices

Chapter ⟨161⟩ assigns the endotoxin limit for medical devices as 20 EU/device except for those medical devices that come in contact with the cerebrospinal fluid, which has an assigned limit of 2.15 EU/device. Devices that contact the anterior segment of the eye should not exceed a limit of 0.2 EU/mL or 0.2 EU/device, as appropriate. Endotoxins in or on solid matrix medical devices are not measured directly, but rather the device is rinsed, soaked, or extracted in an appropriate volume of solvent (generally *WFI*) and the extracts are tested; in some cases extracts from several devices are pooled for testing. As a result, the endotoxin limit for a device extract is expressed in EU/mL, which can later be converted mathematically to EU/device. The endotoxin limit for an extract is inversely proportional to the volume of solvent used for the extraction. The relationship is:

$$\text{Endotoxin Limit} = \frac{K \times N}{V}$$

K = where endotoxin limit per device (20 EU unless otherwise defined and justified; 2.15 EU/device for IT devices)
N = number of devices represented in the pool
V = total volume of solvent used to extract the devices

For example, if the laboratory tests 10 IT devices, each with an extraction volume of 50 mL, the endotoxin limit specification for the pooled extract is:

$$\text{Endotoxin Limit} = \frac{K \times N}{V} = \frac{(2.15\ \text{EU/device}) \times (10\ \text{devices})}{500\ \text{mL}} = 0.04\ \text{EU/mL}$$

[NOTE—Although primary packaging components such as vials or stoppers may be tested using the techniques described in ⟨161⟩, they are not considered to be medical devices. The laboratory should assign endotoxin limits to these components that are much lower than the limits for standard medical device, but appropriate for the drug formulation and presentation. Also some medical devices are liquid (e.g., dialysis fluid) or a solid (e.g., an enzyme). For these products, the endotoxin limit is calculated or assigned and tested as if the device were a drug.]

Maximum Valid Dilution

As product-associated interferences are diluted, so will any endotoxins in the sample be diluted. Therefore a calculation called the MVD is included in the compendial chapter to define the upper bound of allowable product dilution. The MVD is dependent on the endotoxin limit for the product, the starting concentration of the product (generally the concentration of the active ingredient), and the sensitivity of the test method. The MVD is defined as:

$$\text{MVD} = \frac{(\text{Endotoxin Limit}) \times (\text{Product Concentration})}{\lambda}$$

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where

- The endotoxin limit is the calculated limit for the product or device.
- The product concentration equals the concentration of the active ingredient in units per milliliter. For those products administered on a milliliter per kilogram basis or for medical device extracts, the product concentration equals 1.
- λ = the confirmed label claim sensitivity for gel-clot method or the lowest point on the referenced standard curve for the quantitative tests

Additional information on the MVD includes the following:

- The endotoxin limit is constant for any given formulation/dose/administration.
- The MVD is directly related to the starting concentration of active ingredient, and the higher the starting concentration, the higher the MVD.
- The MVD is inversely related to the numerical value given to the test method sensitivity. The more sensitive the test (λ as the denominator in the MVD formula gets lower), the higher the MVD. Changing the test sensitivity to a lower number (more sensitive test) will assist in providing additional dilution room for interfering products.
- The units in the formula cancel out. The resulting calculated value is a dilution factor with no units. For example, an MVD of 100 means that maximum dilution for the product concentration can be diluted no further than 1:100 to have a valid test.
- The MVD does not limit the necessary product dilution when determining the true amount of product contamination in a sample that fails to meet the endotoxin limit. When a product fails at the MVD, it does not meet the endotoxin limit requirement. In this case, it is recommended that the laboratory determine the total bacterial endotoxin content in that product by dilution to extinction (a negative test for gel clot and a valid reading on the quantitative standard curve). The actual level of endotoxin contamination in the product may prove very helpful for trending purposes and to determine the root cause of the contamination during the OOS investigation.

Method Suitability Testing

INTERFERENCE SCREENING FOR DRUG PRODUCTS, INCLUDING BIOLOGIC DRUGS

The principles and practice for performing inhibition/enhancement testing are provided in ⟨85⟩, *Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors* or ⟨85⟩, *Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors*. Although historically suitability tests utilizing three consecutive lots of drug product were considered sufficient to assess suitability, it is recommended that an appropriate number of lots of product be determined prospectively for suitability testing to enable a valid assessment for the potential of lot-to-lot variability in endotoxins content. This is especially important for biological products or products where product development and process validation has indicated significant lot-to-lot variability. Products with greater variability in their starting material, API, and manufacturing process will typically require more than three lots for suitability testing, whereas for products with little or no process or product variability, three lots may suffice. The number of lots tested for suitability should be supported by a risk assessment including information on the life cycle stage of the product (clinical/commercial), known sources of variability, and material testing history that could support an increased or decreased number of lots chosen for suitability testing. All materials that are being tested using methods described in ⟨85⟩ including excipients and raw materials, should have a suitability study to assure that any interferences and variability are identified, mitigated (if necessary) and taken into account in the testing procedures.

COMMON TEST INTERFERENCES

Most pharmaceutical products have been found to interfere to some extent with BET performance (*18*). Because of the high assay sensitivity, these product-specific interferences can usually be overcome by dilution in Water for BET [see *Bacterial Endotoxins Test* ⟨85⟩, *Reagents and Test Solutions, Water for Bacterial Endotoxins Test (BET)*], not to exceed the product-specific MVD. Interferences may affect either the enzyme cascade of the LAL reaction itself or the analyte used as the PPC (e.g., purified LPS), standard, or both. *Table 3* is a listing of common interferences and mitigations that may be considered and implemented beyond mere dilution (*19*). Where buffers or solvents other than Water for BET are used for dilution they should be free of detectable endotoxins.

Table 3. Common Interferences and Mitigations

Interference	Interferes With	Mitigation
pH	LAL cascade	Dilution in Water for BET Adjust pH of the product with hydrochloride, sodium hydroxide, or dilution in tris buffer so that lysate + sample is within the optimal range specified by the lysate manufacturer.
Osmolarity	LAL cascade	Dilution in Water for BET is usually sufficient.
Chelating agents	LAL cascade LPS aggregation	Dilution in Water for BET Add a magnesium ion-containing buffer.
Glucan	LAL cascade	Use a glucan blocker supplied by the reagent manufacturer.

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Table 3. Common Interferences and Mitigations *(continued)*

Interference	Interferes With	Mitigation
Nonspecific protein interference (e.g., serine proteases)	LAL cascade	Dilution in Water for BET Dilution in *Water for BET* combined with heat
Heavy metals	LAL cascade LPS aggregation	Dilution in Water for BET or diluents containing 1 mM chelating agent ethylenediaminetetraacetic acid (EDTA)
Proteins	LAL cascade LPS aggregation	Dilution in Water for BET or normal saline Dilution in Water for BET combined with heat
High detergent	LAL cascade LPS aggregation	Dilution in Water for BET is usually sufficient.
Calcium cation	LAL cascade	Dilution in Water for BET or diluents containing 1 mM chelating agent EDTA

A majority of test interferences are overcome by simple dilution. Dilution can be in Water for BET, buffers (e.g., tris or HEPES), buffers containing glucan-blocking agents, and buffers containing divalent cations or chelating agents as required. Dispersing agents may be added to diluents to further mitigate interference, and their value as an additive is typically considered during method development. It is noteworthy that glucan interference is a common interference in some biological products. Where glucan interference is likely, the use of a glucan blocker should be considered during assay suitability test method development. Diluents other than Water for BET and common buffers described above should be checked to assure that they do not interfere with the BET assay. For example, some neutralizing agents (e.g., proteinase K) may not be manufactured with appropriate controls against their contamination with GNB. If these reagents are used, they should be checked to assure that they do not contain endotoxins at levels that may contribute to the levels ultimately reported for the product. All BET assays must be conducted at neutral pH. Neutral pH for BET assays is generally defined as 6.0–8.0, but because all lysates are different in their formulations, the laboratory should confirm the proper range as described in the lysate manufacturer's package insert. However, the reagent itself may provide buffering capacity so the mixture of lysate and product dilution should be tested for pH during the suitability study. pH may be adjusted to neutrality by the use of buffers as diluents or endotoxin-free hydrochloride (HCl) or sodium hydroxide (NaOH). If adjustment of pH is required using acid or base, it is suggested that the pH be measured during the suitability study to assess lot-to-lot variation in the product.

Lysates from different manufacturers have different proprietary formulations, and all are calibrated for sensitivity against the RSE to ensure that they all detect LPS activity equally well in water. However, LPS activity may not be recovered equally in different products, or by using different compendial test methods. If there is significant product-specific interference that cannot be overcome by dilution to within the MVD or by standard mitigation methodologies (*Table 3*), the laboratory should consider a different lysate formulation or test method before concluding that the LAL-bacterial endotoxins test is either invalid or unacceptable (*20*).

Qualifying Test Preparation Methods Other than Dilution

For sample preparation that requires manipulation other than simple dilution, e.g., heating or ultrafiltration, ⟨85⟩ instructs the analyst to proceed as follows:

> Interference may be overcome by suitable treatment such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

ROUTINE TESTING

Sampling

All materials used to sample materials for endotoxin content (scoops, pipettes, bottles) must be inert with respect to the material(s) being sampled, and they must be sterile and free of detectable endotoxins. Even though samples are often taken in the field without the aid of a laminar flow hood, samplers must take precautions that a) they do not contaminate the sample itself, and b) in taking the sample, they do not contaminate the rest of the material in its original container.

Historically, the sampling scheme for finished drug products is to take at least 3 units representing the beginning, middle, and end of the batch. However, for a standard run of a small-volume parenteral, biological therapy, or large-volume parenteral, 3 units may not be a representative sample and may identify only those lots that are uniformly and highly contaminated. Sampling schemes should be justified and should be based on the known variability of the process, the unit operations of the process, historical knowledge of the process, and materials used in manufacture.

Bacteria and endotoxins are generally not homogeneously distributed in any product or material, liquid or powder. Care must be taken to flush ports in a circulating water loop before sampling and use the same equipment for sampling that is used for manufacture to assure a representative sample. For example, if a hose is attached to a port for the purpose of transferring water from a circulating loop to another a formulation vessel, the water sample must be taken from the same hose with the same flush times as used by manufacturing. So, while the water within the a properly constructed and controlled loop may theoretically be homogeneous, differences in the ports along the distribution loop may add endotoxins to samples.

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For highly viscous materials, a suitable inert rod free of detectable endotoxins may be used to mix the material prior to sampling. Sterile, pyrogen-free spatulas and scoops are used for powdered and granular solids. Because of the lack of assurance of homogeneity, the number of samples from viscous materials and powders should be determined relative to the number of units initially received using appropriate statistical procedures.

Particularly for samples of viscous and powdered materials, samplers should have training on how to spot signs of non-uniformity. Such indicators may include differences in shape, size, or color or evidence of moisture in powdered materials. For viscous materials, samplers should be trained to look for stratification of the material, differences in viscosity or colors, and particulate contamination. Samples of raw materials that could exhibit variability in endotoxin content should not be pooled (see *Pooling*).

In the case of a new vendor of a raw material, the methodology and calculations used in the determination of the endotoxin content of the material should be reviewed. If the material is designated as critical for manufacturing, a site audit of the vendor may be in order. In addition, it is highly suggested that an in-house confirmation of the accuracy of the endotoxin level stated on the CoA be performed. For critical materials, the quality agreement should require that manufacturers of these materials inform the pharmaceutical manufacturer of changes in processes, controls, or raw materials so that the changes can be discussed at change control and the need for re-confirmation of materials can be determined. Once the CoAs have been successfully confirmed for a predetermined number of shipments justified in the sampling plan by history, and depending on the variability of the manufacturing process, the laboratory can consider accepting the CoA with periodic testing of the incoming materials, as long as the manufacture of the materials has not changed.

Pooling

The term "pooling" refers to creating a composite sample preparation that includes the total contents of several individual units or equal aliquots from the units taken from the same lot or batch. Pooling is often done for batch-release testing. Pooling is an acceptable option for laboratories performing any of the BET assays on drug products (*9*). However, pooling has a number of drawbacks; the most obvious is that it will dilute endotoxins that may be in any one of the units, potentially concealing the variability in endotoxin content and distribution among the units that were pooled. Sampling plans, including instructions to pool samples, should be scientifically justified.

If units are pooled, the MVD must be adjusted to account for the possibility of endotoxins in just one of the samples (*9*). The adjusted MVD is calculated by dividing the originally calculated MVD by the number of units contributing to the pool. For example, if the MVD for a small volume parenteral is 240 and a manufacturer chooses to pool three vials for testing, the adjusted MVD is 240/3, which is 80. This reduction in MVD effectively reduces the endotoxin limit for the product by a factor of three to compensate for pooling. While pooling may result in an incremental savings in reagent usage and therefore save some money, there are a number of points to consider when pooling:

- Pooling may obscure any non-uniformity in endotoxin content between the individual sample units. Information on variability may be valuable in troubleshooting or investigations. For example, random contamination in one of multiple filling needles may cause some vials to contain endotoxins and others not.
- Taking aliquots of samples for pooling should always be performed using aseptic technique and with individual units vigorously mixed prior to removing the aliquots. The original containers with remaining product should be retained for investigation in the event of an OOS test result. Removing the aliquot through the disinfected rubber stopper using a pyrogen-free syringe is advisable to maintain the integrity of the unit container during subsequent storage for investigative testing.
- The concept of adjusted MVD does not apply to medical devices as they are, by convention, commonly pooled for testing.
- Any sampling scheme for drug products must represent the beginning, middle, and end of the batch. Additional samples may be taken if interventions in manufacturing raise concerns about possible endotoxin contamination.
- If testing at the adjusted MVD causes an unacceptable increase in product-specific interference, samples should be tested individually.
- Products with low calculated MVDs, or suspensions where there is no assurance of homogeneity in the removed aliquots, may not be good candidates for pooling.
- Pooling is not appropriate for in-process samples, particularly those representing different stages of manufacturing.

Calculation of Endotoxin Content

All BET results are provided in EU/mL. If the product being tested has a limit expressed in EU per unit of weight or activity (e.g., EU/mg), a calculation must be made to convert EU/mL to EU/mg. For example, the endotoxin content for a product with a starting concentration of 10 mg/mL, after adjustment for the dilution factor, is determined by gel clot to be 5 EU/mL. The endotoxin content expressed in EU/mg is:

$$(5\ \text{EU/mL}) \div (10\ \text{mg/mL}) = 0.5\ \text{EU/mg}$$

Out-of-Specification Results and Retesting Considerations

An endotoxins test result that does not meet product specifications necessitates an investigation (*21*). The suspect result is considered valid unless a comprehensive investigation clearly demonstrates otherwise.

Invalid tests, however, are not OOS results. Invalid tests are those where system suitability parameters such as negative controls, PPCs, confirmation of label claim in gel clot, or generation of a linear standard curve do not function as expected and

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therefore may affect the accuracy of the test results. Although invalid tests should be tracked and trended to look for patterns and trends that might require a corrective action, a true OOS test failure only exists when a valid assay has been performed and generates results that exceed the specification.

A finding of a nonconforming test result requires a laboratory assessment to assure the accuracy of the data. The FDA OOS guidance for industry terms this a Phase I investigation (*21*). Despite the fact that a failed BET assay is rare, the investigation should be done in a timely manner to ascertain if there was anything atypical about this sample, its preparation, calculations, or performance of the test that may affect the accuracy of the test result. Phase I should include a critical and in-depth review of sampling methods and techniques, sample hold conditions, sample preparation, test method parameters, and any documentation, including real-time notations of testing errors by analysts and a review to find any errors in calculation. An organizing tool, such as a fault tree analysis (FTA) or Ishikawa (fishbone) diagram, may help the team members to organize their thoughts and complete the investigation in a timely and compliant manner.

The use of a checklist approach will promote completeness and consistency across all assessments.

The purpose of the Phase I laboratory investigation is to confirm the accuracy of the original test result. A Phase I checklist should include the following, at a minimum:

- The testing history of the material under test: Has a specific assay problem or repeated failures for the same product been previously observed?
- Sampling procedures, sample container integrity, and sample preparation: Were samples taken, transported, and stored correctly, following approved methods?
- Reagent preparation, storage, and use.
- Calibration and maintenance status of dispensing, incubation, and reading instrumentation.
- Appropriateness of sample contact equipment and disposables.
- The test procedure: Is it consistent with the suitability data, and was it precisely followed?
- Were all controls and other system suitability indicators within normal limits? If not, then the test is not OOS and is invalid.
- Were all calculations (product endotoxin limits, MVD, and endotoxin contamination levels) performed correctly? Were all transcriptions performed accurately?
- Was the analyst adequately trained? What is the analyst's OOS history?

An approved BET standard operating procedure (SOP) should include two important directions to analysts:

1. If an analyst realizes and documents that a mistake has been made during the testing, including an error in calculation, sample preparation, dilution, or performance of the assay that could affect the accuracy of the test results, the test should be stopped and the reason for the termination of the test documented at the time that the error was identified.
2. Analysts should not discard the original sample, any of the sample preparation dilution tubes, or the reagents used until the results of the assay are known and evaluated against the product specification. The tubes and reagents from an OOS assay may be an important part of the laboratory investigation.

If there is no analytical failure or documentation error found, the result is considered valid and the product is OOS. On the other hand, if the analysis was conducted incorrectly and documented error(s) are the root cause of the failure, the initial assay is invalid and the product may be tested again with appropriate controls.

Investigation of production practices, otherwise known as Phase II investigations, require assessment of all manufacturing activities that could have impacted the endotoxin content of the failed product sample. Phase II may be more easily conducted by a cross-functional team including operations, facilities, and engineering, with assistance from a microbiologist to identify potential sources of contamination relating to contamination control requirements during manufacturing.

The Phase II investigation is actually a reassessment of validated process control, particularly if the failure was not the first failure for the product. A product manufactured under a state of control should not fail endotoxins testing, so the Phase II investigation should focus on anything that may have been different about the lot in question.

At any point in the investigation, testing can be performed to challenge hypotheses regarding the reason for the failure of the material to meet its limit. However, it is important to note that these investigational tests are not considered to be re-tests and should not be used to release material. Rather, they are used to identify conditions that might have contributed to an inaccuracy in the testing of the product. A laboratory SOP or policy should also be written that precisely describes the procedure(s) that must be followed for the purposes of investigational testing. Such a plan eliminates situational decisions regarding the appropriateness of investigational testing, retesting, or resampling; the plan should be approved by the quality unit.

Investigational testing should be well-defined and justified, pre-approved, and well-documented. If an investigational second test of the original sample preparation finds that the product meets the specifications for the test, it is possible that an error was made in some aspect of the execution of the original assay or just as easily in the execution of the second test. If this is the case, any hypotheses should be proposed and testing executed to confirm that an error was made. The intended purpose of the investigational tests on the sample is not to reverse the original OOS result but rather to gain information regarding a potential failure so that a more effective effort can be made to pinpoint the problem and implement any necessary corrective and preventive actions.

The statement in ⟨85⟩ that reads "In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested" has been widely misinterpreted and is relevant *only* to the specific case of a conflicting result between a regulatory agency using the gel-clot method for lot release testing and a testing laboratory using a different LAL-bacterial endotoxins test platform. It is not intended to allow a laboratory to use a gel-clot result that provides passing results when the laboratory's customary assay method for the product shows an OOS test result.

In the event of a product failure, the total level of endotoxins activity in the failed sample(s) should be determined. For gel-clot assays, this may involve testing of dilutions beyond the MVD until an endpoint is reached for gel clot or a valid test result is

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obtained for quantitative tests. These values may provide a clue as to the source of potential or actual product contamination, or a case of mishandling of a positive control, or a tube mix-up.

Standard Curve Control

Standard curves are created for endpoint chromogenic assay by evaluating the direct relationship between color intensity and standard concentration. The resulting curve has a positive slope, and ⟨85⟩ indicates that the correlation coefficient of the curve must be ≥0.980.

The standard curve is created by plotting the $\log_{10}$ of the onset or reaction time required for each standard to reach a predetermined optical density for kinetic turbidimetric assays or color intensity for kinetic chromogenic assays, as a function the $\log_{10}$ of the standard concentration. This transformation of the data results in a standard curve with a negative slope, and the correlation coefficient must be ≤ –0.980. Therefore, it can be stated that the linearity requirement for all compendial quantitative BET assays is $|r| \geq 0.980$. The requirement is expressed to three significant figures because the last "0" is important; it is inappropriate to have a standard curve with $|r| = 0.979$ and round up. However, in a well-controlled laboratory, correlation coefficients should routinely be greater than 0.980.

All standard curves have a corresponding linear equation $y = mx + b$ where m is the slope of the standard curve regression line and b is the y-intercept. Because the y-intercept is the point where $x = 0$, and because in a kinetic assay the $\log_{10}$ of 1 is 0, the y-intercept is really at the 1 EU standard. Because of the transformation of data to $\log_{10}$, standard curves for quantitative assays can be very sensitive to small changes in onset times.

Accuracy of test results depends on the accuracy of the standard curve. Therefore, the onset times for standards representing unique combinations of lysate lot and CSE lot from run to run, instrument to instrument and analyst to analyst should all be monitored. For example, an analyst can make a twofold or tenfold dilution error in the dilution of the standards, yet still produce a linear standard curve that meets the requirement $|r| \geq 0.980$. The dilution error would not be noticed by looking at the correlation coefficient alone, but would be evident in the onset times of the standards. An overdiluted set of standards would run more slowly (longer onset/reaction times) than a properly diluted standard series, and an underdiluted set of standards would run more quickly (shorter onset/reaction times) than a properly diluted standard series. The dilution error would also be reflected in the values generated for the y-intercept. Changes in slope can also affect the accuracy of the test data. It is suggested that part of an analyst's training in quantitative assay performance is to understand the impact of variability on the accuracy of the test result (*22*,*23*).

ALTERNATE TEST METHODS

The methods listed in ⟨85⟩ for the detection of bacterial endotoxins (gel-clot limits test, gel-clot assay, kinetic chromogenic, endpoint chromogenic, kinetic turbidimetric) are considered to be validated. However, a laboratory may choose to use an assay methodology that is not listed in ⟨85⟩. If such a choice is made, the alternate test for the detection of bacterial endotoxins must be fully validated to ensure that decisions made using the alternate methodology are equivalent to or better than decisions made using the validated USP methods and ultimately approved by the appropriate regulatory authority. Although endotoxin testing is not specifically cited, guidance on how to think about the validation of alternate methods can be found in *Validation of Alternative Microbiological Methods* ⟨1223⟩ and *Validation of Compendial Procedures* ⟨1225⟩.

GLOSSARY

Bacterial endotoxins: A GNB outer membrane macromolecular complex of polysaccharide, lipid, and protein. Extracted, purified, and highly concentrated (protein free) endotoxin is generally referred to as lipopolysaccharide or LPS to distinguish it from the more natural complexed cell membrane associated form (*24*).

Bacterial endotoxins test (BET): Compendial bacterial endotoxins test

Control standard endotoxin (CSE): A preparation with a stable endotoxin concentration calibrated against RSE. CSEs are generally prepared by vendors or by laboratories, and although called "standards," are not certified for either purity or activity by a third party. CSEs are best described as calibration analytes. CSEs may be purified or they may be a preparation of native endotoxins. If a CSE is a preparation not already adequately characterized, its evaluation should include characterizing parameters such as activity, uniformity, and stability, which would demonstrate the suitability of the material to serve in the calibration of a BET. Detailed procedures for its preparation and use to ensure consistency in performance should also be included.

Intrathecal: A parenteral injection that results in the product coming in contact with the cerebrospinal fluid

Lysate or limulus amebocyte lysate (LAL): The reagent used in the performance of the BET

Parenteral: Drugs or medical devices that are injected, infused, or implanted or that may otherwise come in contact with the bloodstream or cerebrospinal fluid

USP Endotoxin Reference Standard (RSE): Primary endotoxin standard. RSE is an extracted, purified, and formulated preparation of *E. coli* 0113:H10:K LPS.

Suitability: Demonstration that the chosen assay is appropriate for its defined purpose in testing the material

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▲ (USP 1-Dec-2019)

⟨1086⟩ IMPURITIES IN DRUG SUBSTANCES AND DRUG PRODUCTS

INTRODUCTION

This general information chapter is intended to provide common terminology for impurities and degradation products that may be present in compendial drug substances and drug products. Impurities or degradation products in drug substances can arise during the manufacturing process or during storage of the drug substance. The degradation products in drug products can arise from drug substances or reaction products of the drug substance with the environment, with an excipient, or an immediate container–closure system. Biological and biotechnological products, fermentation products and semisynthetic products derived therefrom, and radiopharmaceutical products are not covered in this chapter.

Communications about impurities and degradation products in compendial articles may be improved by including in this Pharmacopeia the definitions of terms and the contexts in which these terms are used. (See *Glossary* below.) There has been

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much activity and discussion in recent years about the definition of terms. Certain industry-wide concerns about terminology and context deserve widespread publication and ready retrievability and are included here. See *General Notices, 5.60 Impurities and Foreign Substances*, as well as the general chapter *Ordinary Impurities* ⟨466⟩. Some other general chapters added over the years have also addressed topics of purity or impurity as these have come into focus or as analytical methodology has become available. Analytical aspects are enlarged upon in the chapter *Validation of Compendial Procedures* ⟨1225⟩.

Purity or impurity measurements for drug products present a challenge to Pharmacopeial standards-setting. Where degradation of a drug product over time is at issue, the same analytical methods that are stability-indicating are also purity-indicating. Resolution of the active ingredient(s) from the excipients necessary to the preparation presents the same qualitative problem. Thus, many monographs for Pharmacopeial preparations feature chromatographic assays. Where more significant impurities are known, some monographs set forth specific limit tests. In general, however, this Pharmacopeia does not repeat impurity tests in subsequent preparations where these appear in the monographs of drug substances and where these impurities are not expected to increase. It is presumed that adequate retention specimens are in storage for the exact batch of drug substances used in any specific lot of a drug product. Whenever analysis of an official article raises a question of the official attributes of any of the drug substances used, subsequent analysis of retention specimens is in order.

DRUG SUBSTANCE

Classification of Impurities

Impurities can be classified into the following categories:

1. Organic impurities (process- and drug-related)
2. Inorganic impurities
3. Residual solvents

Organic impurities can arise during the manufacturing process and/or storage of the drug substance. They can be identified or unidentified, volatile or nonvolatile, and include the following:

1. Starting materials
2. Byproducts
3. Intermediates
4. Degradation products
5. Reagents, ligands, and catalysts
6. Geometric and stereoisomers

Inorganic impurities can result from the manufacturing process. They are normally known and identified and include the following:

1. Reagents, ligands, and catalysts
2. Heavy metals or other residual metals
3. Inorganic salts
4. Other materials (e.g. filter aids, charcoal)

Residual solvents are organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of a drug substance. Because these are generally of known toxicity, the selection of appropriate controls is easily accomplished (see *Residual Solvents* ⟨467⟩).

Concepts for setting impurity or degradation product limits in drug substances are based on chemistry and safety concerns. As such, limits for organic and inorganic impurities and residual solvents should be established for drug substances. The basic tenet for setting limits is that levels of impurities or degradation products in a drug substance must be controlled throughout its development to ensure its safety and quality for use in a drug product.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

DRUG PRODUCT

The specification for a drug product should include a list of degradation products expected to occur during manufacture of the commercial product and under recommended storage conditions. Stability studies, knowledge of degradation pathways, product development studies, and laboratory studies should be used to characterize the degradation profile. The selection of degradation products in the drug product specification should be based on the degradation products found in batches manufactured by the proposed commercial process.

This rationale should include a discussion of the degradation profiles observed in the safety and clinical development batches and in stability studies, together with a consideration of the degradation profile of batches manufactured by the proposed commercial process. For degradation products known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation/detection limit of the analytical procedures should be commensurate with the level at which the degradation products should be controlled.

For drug products the concept for setting degradation product limits is based on sound scientific judgment as applied to available data on the safety and stability of the drug product, data that may include the degradation pathways of the drug substance, the manufacturing process, known excipient interactions, any safety assessment studies, stability studies conducted under the recommended storage conditions, and ancillary studies that may provide additional information on the stability profile of the drug product. Impurities that are not degradation products (e.g., process impurities from the drug substance) are often not controlled in the drug product, as they are typically controlled in the drug substance and these impurities are not

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expected to increase over time. Additional guidance for setting limits can be found in various ICH and FDA guidance documents, as well as in the USP monograph submission guidelines.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

Drug products should contain levels of residual solvents no higher than can be supported by safety data (see *Residual Solvents* ⟨467⟩).

GLOSSARY

Concomitant components: Concomitant components are characteristic of many drug substances and are not considered to be impurities in the Pharmacopeial sense. Limits on contents, or specified ranges, or defined mixtures are set forth for concomitant components in this Pharmacopeia. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

Degradation product: An impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and/or the immediate container–closure system.

Foreign substances (extraneous contaminants): An impurity that arises from any source extraneous to the manufacturing process and that is introduced by contamination or adulteration. These impurities cannot be anticipated when monograph tests and assays are selected. The presence of objectionable foreign substances not revealed by monograph tests and assays constitutes a variance from the official standard. Examples of foreign substances include ephedrine in Ipecac or a pesticide in an oral liquid analgesic. Allowance is made in this Pharmacopeia for the detection of foreign substances by unofficial methods. (See *General Notices, 5.60 Impurities and Foreign Substances.*)

Identified impurities and identified degradation products: Impurities or degradation products for which structural characterizations have been achieved.

Impurity: Any component of a drug substance that is not the chemical entity defined as the drug substance and in addition, for a drug product, any component that is not a formulation ingredient.

Inorganic impurities: Inorganic impurities can result from the manufacturing process (e.g., residual metals, inorganic salts, filter aids, etc.). Inorganic impurities are typically controlled by tests such as *Residue on Ignition* ⟨281⟩. Information found in *Plasma Spectrochemistry* ⟨730⟩ and *Ion Chromatography* ⟨1065⟩ may also be of value.

Intermediate: A material that is produced during steps of the synthesis of a drug substance and that undergoes further chemical transformation before it becomes a drug substance. The intermediate is often isolated during the process.

Ordinary impurities: Some monographs make reference to ordinary impurities. For more details see *Ordinary Impurities* ⟨466⟩.

Other impurities: See section *5. Monograph Components* under *General Notices and Requirements.*

Polymorphs: Different crystalline forms of the same drug substance. These can include solvation or hydration products (also knows as pseudopolymorphs) and amorphous forms. Although polymorphs are not impurities by definition, an understanding of the crystalline forms, hydration or solvation states, or amorphous nature is critical to the overall characterization of the drug substance.

Process contaminants: Process contaminants are identified or unidentified substances (excluding related substances and water), including reagents, catalysts, other inorganic impurities (e.g., heavy metals, chloride, or sulfate); and may also include foreign substances (extraneous contaminants). These contaminants may be introduced during manufacturing or handling procedures.

Reagent: A substance other than a starting material, intermediate, or solvent that is used in the manufacture of a drug substance.

Related substances: Related substances are structurally related to a drug substance. These substances may be (a) identified or unidentified impurities arising from the synthesis manufacturing process, such as starting materials, intermediates, or by-products, and do not increase on storage, or (b) identified or unidentified degradation products that result from drug substance or drug product manufacturing processes or arise during storage of a material.

Residual solvents: An organic liquid used as a vehicle for the preparation of solutions or suspensions in the synthesis of a drug substance (see *Residual Solvents* ⟨467⟩).

Specified impurities and specified degradation products: Previously referred to as Signal Impurities, specified impurities or specified degradation products are impurities or degradation products that are individually listed and limited with specific acceptance criteria in individual monographs as applicable. Specified impurities or specified degradation products can be identified or unidentified.

Starting material: A material that is used in the synthesis of a drug substance and is incorporated as an element into the structure of an intermediate and/or of the drug substance. Starting materials are often commercially available and have well-defined chemical and physical properties and structure.

Stereomeric impurity: A compound with the same 2-dimensional chemical structure as the drug substance but differs in the 3-dimensional orientation of substituents at chiral centers within that structure. In those cases where all chiral centers are in the opposite orientation, the impurity is an enantiomer (enantiomeric impurity). Determinations of impurities in this category often require special chiral chromatographic approaches. Diastereomeric or epimeric impurities occur when only some of the chiral centers are present in the opposite orientation.

Toxic impurities: Toxic impurities have significant undesirable biological activity, even as minor components, and require individual identification and quantification by specific tests. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. Based on validation data, individualized tests and specifications are selected. These feature comparison to a Reference Standard of the impurity, if available. It is incumbent on the manufacturer to provide data that would support the classification of such impurities as toxic impurities.

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Unidentified impurities and unidentified degradation products: Impurities or degradation products for which structural characterizations have not been achieved and that are identified solely by qualitative analytical properties (e.g., chromatographic retention times).

Unspecified impurities and unspecified degradation products: Impurities or degradation products that are limited by general acceptance criteria but not individually listed with their own specific acceptance criteria in individual monographs.

⟨1087⟩ APPARENT INTRINSIC DISSOLUTION—DISSOLUTION TESTING PROCEDURES FOR ROTATING DISK AND STATIONARY DISK

This general information chapter *Apparent Intrinsic Dissolution—Dissolution Testing Procedures for Rotating Disk and Stationary Disk* ⟨1087⟩ discusses the determination of dissolution rates from nondisintegrating compacts exposing a fixed surface area to a given solvent medium. Compact, as used here, is a nondisintegrating mass resulting from compression of the material under test using appropriate pressure conditions. A single surface having specified physical dimensions is presented for dissolution. Determination of the rate of dissolution can be important during the course of development of new chemical entities because it sometimes permits prediction of potential bioavailability problems and may also be useful for characterizing compendial articles such as excipients or drug substances. Intrinsic dissolution studies are characterization studies and are not referenced in individual monographs. Information provided in this general information chapter is intended to be adapted via a specific protocol appropriate to a specified material.

Dissolution rate generally is expressed as the mass of solute appearing in the dissolution medium per unit time (e.g., mass sec^{-1}), but dissolution flux is expressed as the rate per unit area (e.g., mass cm^{-2} sec^{-1}). Reporting dissolution flux is preferred because it is normalized for surface area and, for a pure drug substance, is commonly called intrinsic dissolution rate. Dissolution rate is influenced by intrinsic solid-state properties such as crystalline state, including polymorphs and solvates, as well as degree of noncrystallinity. Numerous investigational approaches are available for modifying the physicochemical properties of chemical entities so that their solubility and dissolution properties are enhanced. Among these approaches are the use of coprecipitates and the use of amorphous solid dispersions. The effect of impurities associated with a material can also significantly alter its dissolution properties. Dissolution properties are also influenced by extrinsic factors such as surface area, hydrodynamics, and dissolution medium properties, including solvent (typically water), presence of surfactants, temperature, fluid viscosity, pH, buffer type, and buffer strength.

Rotating disk and stationary disk dissolution procedures are sufficiently versatile to allow study of the characteristics of compounds of pharmaceutical interest under a variety of test conditions. Characteristics common to both apparatuses include the following:

1. They are adaptable to use with standard dissolution testing stations, and both use a tablet die to hold the nondisintegrating compact during the dissolution test.
2. They rely on compression of the test compound into a compact that does not flake or fall free during the dissolution test.
3. A single surface of known geometry and physical dimension is presented for dissolution.
4. The die is located at a fixed position in the vessel, which decreases the variation of hydrodynamic conditions.

A difference between the two procedures is the source of fluid flow over the dissolving surface. In the case of the rotating disk procedure, fluid flow is generated by the rotation of the die in a quiescent fluid, but fluid flow is generated by a paddle or other stirring device for the stationary disk procedure.

EXPERIMENTAL PROCEDURE

The procedure for carrying out dissolution studies with the two types of apparatus consists of preparing a nondisintegrating compact of material using a suitable compaction device, placing the compact and surrounding die assembly in a suitable dissolution medium, subjecting the compact to the desired hydrodynamics near the compact surface, and measuring the amount of dissolved solute as a function of time.

Compacts are typically prepared using an apparatus that consists of a die, an upper punch, and a lower surface plate fabricated out of hardened steel or other material that allows the compression of material into a nondisintegrating compact. An alternative compaction apparatus consists of a die and two punches. Other configurations that achieve a nondisintegrating compact of constant surface area also may be used. The nondisintegrating compact typically has a diameter of 0.2–1.5 cm.

Compact Preparation

Attach the smooth lower surface plate to the underside of the die, or alternatively, insert the lower punch using an appropriate clamping system. Accurately weigh a quantity of material necessary to achieve an acceptable compact, and transfer to the die cavity. Place the upper punch into the die cavity, and compress the powder on a hydraulic press at a compression pressure required to form a nondisintegrating compact that will remain in the die assembly for the length of the test. Compression for 1 minute at 15 MPa usually is sufficient for many organic crystalline compounds, but alternative compression conditions that avoid the formation of capillaries should be evaluated. For a given substance, the compact preparation, once optimized is standardized to facilitate comparison of different samples of the substance.

Changes in crystalline form may occur during compression; therefore, confirmation of a solid state form should be performed by powder X-ray diffraction or other techniques. Remove the surface plate or lower punch. Remove loose powder from the surface of the compact and die by blowing compressed air or nitrogen over the surface.

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Dissolution Medium

The choice of dissolution medium is an important consideration. Whenever possible, testing should be performed under sink conditions to avoid artificially retarding the dissolution rate due to approach of solute saturation of the medium. Dissolution measurements are typically made in aqueous media. To approximate in vivo conditions, measurements may be run in the physiological pH range at 37°. The procedure when possible is carried out under the same conditions that are used to determine the intrinsic solubility of the solid state form being tested. Dissolution media should be deaerated immediately before use to avoid air bubbles forming on the compact or die surface.[1]

The medium temperature and pH must be controlled, especially when dealing with ionizable compounds and salts. In the latter cases, the dissolution rate may depend strongly on the pH, buffer species, and buffer concentration. A simplifying assumption in constant surface area dissolution testing is that the pH at the surface of the dissolving compact is the same as the pH of the bulk dissolution medium. For nonionizable compounds, this is relatively simple because no significant pH dependence on dissolution rate is expected. For acids and bases, the solute can alter the pH at and near the surface of the compact as it dissolves. Under these conditions, the pH at the surface of the compact may be quite different from the bulk pH due to the self-buffering capacity of the solute. To assess intrinsic solubility, experimental conditions should be chosen to eliminate the effect of solute buffering, alteration of solution pH, and precipitation of other solid state forms at the surface of the compact. For weak acids, the pH of the dissolution medium should be 1–2 pH units below the pKa of the dissolving species. For weak bases, the pH of the dissolution medium should be 1–2 pH units above the pKa of the dissolving species.

Apparatus

ROTATING DISK

A typical apparatus (*Figure 1*) consists of a punch and die fabricated out of hardened steel. The base of the die has three threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compacted pellet. The die has a cavity into which is placed a measured amount of the material whose intrinsic dissolution rate is to be determined. The punch is then inserted in the die cavity and the test material is compressed with a hydraulic press. [NOTE—A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test.] A compacted pellet of the material is formed in the cavity with a single face of defined area exposed on the bottom of the die.

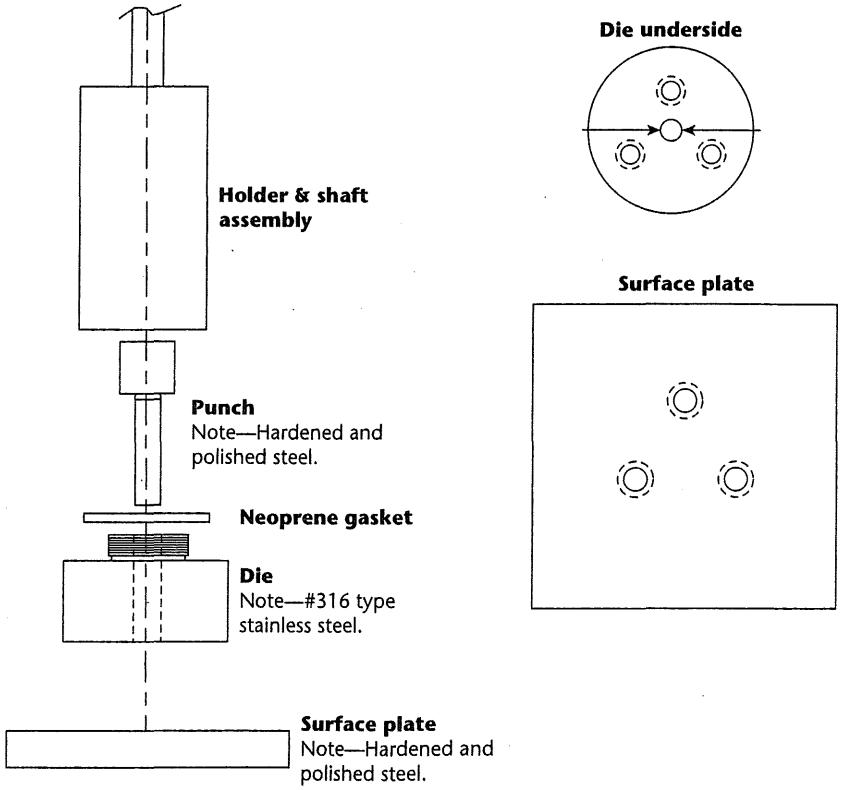

Figure 1

[1] One method of deaeration is the following: Heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter of 0.45-µm or less pore size, with vigorous stirring, and continue stirring under vacuum for about 5 minutes. Other deaeration techniques for removal of dissolved gases may be used.

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The die assembly is then attached to a shaft constructed of an appropriate material (typically steel). The shaft holding the die assembly is positioned so that when the die assembly is lowered into the dissolution medium (*Figure 2*), the exposed surface of the compact will be not less than 1.0 cm from the bottom of the vessel and nominally in a horizontal position. The die assembly should be aligned to minimize wobble, and air bubbles should not be allowed to form on the compact or die surface.

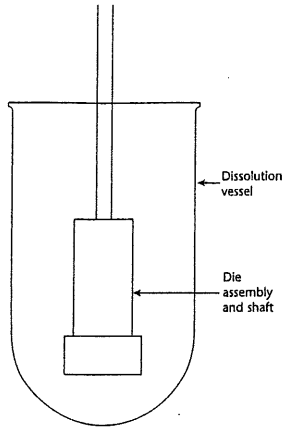

Figure 2

A rotating disk speed of 300 rpm is recommended. Typical rotation speeds may range from 60–500 rpm. The dissolution rate depends on the rotation speed used. This parameter should be selected in order to admit at least five sample points during the test, but excessive stirring speeds may create shear patterns on the surface of the dissolving material that could cause aberrant results (i.e., nonlinearity). Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process. If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

STATIONARY DISK

The apparatus (*Figure 3*) consists of a steel punch, die, and a base plate. The die base has three holes for the attachment of the base plate. The three fixed screws on the base plate are inserted through the three holes on the die and then fastened with three washers and nuts. The test material is placed into the die cavity. The punch is then inserted into the cavity and compressed, with the aid of a bench top press. The base plate is then disconnected from the die to expose a smooth compact pellet surface. A gasket is placed around the threaded shoulder of the die and a polypropylene cap is then screwed onto the threaded shoulder of the die.

The die assembly is then positioned at the bottom of a specially designed dissolution vessel with a flat bottom (*Figure 4*). The stirring unit (e.g., paddle) is positioned at an appropriate distance (typically 2.54 cm) from the compact surface. The die assembly and stirring unit should be aligned to ensure consistent hydrodynamics, and air bubbles should not be present on the compact surface during testing. Alternative configurations may be utilized if adequate characterization and control of the hydrodynamics can be established.

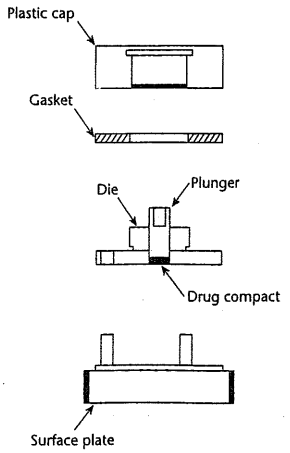

Figure 3

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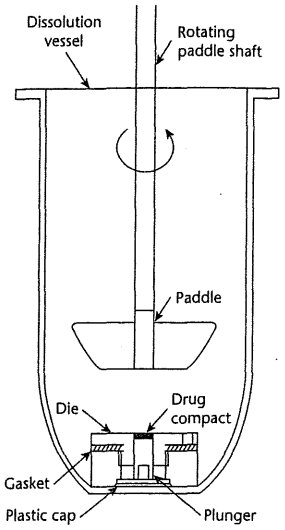

Figure 4

The dissolution rate depends on the rotation speed and precise hydrodynamics that exist. Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process (see *Rotating Disk*). If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

DATA ANALYSIS AND INTERPRETATION

The dissolution rate is determined by plotting the cumulative amount of solute dissolved against time. Linear regression analysis is performed on data points in the initial linear region of the dissolution curve. The slope corresponds to the dissolution rate (mass sec^{-1}). (More precise estimates of slope can be obtained using a generalized linear model that takes into account correlations among the measurements of the cumulative amounts dissolved at the various sampling times.)

The amount versus time profiles may show curvature. When this occurs, only the initial linear portion of the profile is used to determine the dissolution rate. Upward curvature (positive second derivative) of the concentration versus time data is typically indicative of a systematic experimental problem. Possible problems include physical degradation of the compact by cracking, delaminating, or disintegration. Downward (negative second derivative) curvature of the dissolution profile is often indicative of a transformation of the solid form of the compact at the surface or when saturation of the dissolution medium is inadvertently being approached. This often occurs when a less thermodynamically stable crystalline form converts to a more stable form. Examples include conversion from an amorphous form to a crystalline form or from an anhydrous form to a hydrate form, or the formation of a salt or a salt converting to the corresponding free acid or free base. If such curvature is observed, the crystalline form of the compact may be assessed by removing it from the medium and examining it by powder X-ray diffraction or another similar technique to determine if the exposed surface area is changing.

The constant surface area dissolution rate is reported in units of mass sec^{-1}, and the dissolution flux is reported in units of mass cm^{-2} sec^{-1}. The dissolution flux is calculated by dividing the dissolution rate by the surface area of the compact. Test conditions, typically a description of the apparatus, rotation speed, temperature, buffer species and strength, pH, and ionic strength should also be reported with the analyses.

⟨1088⟩ IN VITRO AND IN VIVO EVALUATION OF DOSAGE FORMS

PURPOSE

This chapter provides an overview of the methodology for characterizing the physicochemical properties of a drug substance as well as its associated drug product and discusses the relationship of these methods and properties to the pharmacokinetic and pharmacodynamic properties of the drug product. Results of in vitro methods are linked with information from in vivo evaluations through an in vitro–in vivo correlation (IVIVC).

SCOPE

The ultimate goal of these characterization studies is an understanding of the relationship between the physicochemical and pharmacological properties of the drug substance to the pharmacokinetic properties and in vitro performance of the drug product. This chapter outlines the in vitro and in vivo testing that goes into the development of the body of data that informs decision making relating to the formulation, manufacturing, and related regulatory activities necessary for the development,

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regulatory approval, and marketing of any drug product. The chapter complements the information in general chapters, *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩ and *The Dissolution Procedure: Development and Validation* ⟨1092⟩ by detailing the essential in vitro and in vivo data elements underlying an understanding of bioequivalence and bioavailability. The chapter text recognizes that regulatory guidances and a wealth of text books are available to elaborate on the content provided, and it is not the purpose to provide an exhaustive disquisition on the subjects presented but rather to provide a guide and listing of the issues of interest.

BACKGROUND INFORMATION

Establishing a meaningful relationship between dissolution behavior and in vivo drug performance (i.e., IVIVC) has long been sought from the perspectives of both bioavailability (BA) and bioequivalence (BE) and quality control considerations. In setting dissolution acceptance criteria for a product monograph, USP's policy has been to give predominant consideration to valid BA or BE studies, when available.

The earliest achievable in vitro characteristic thought to predict an acceptable in vivo performance was tablet and capsule disintegration. A test for disintegration was adopted in *USP XIV* (1950). At that time, no quantitative work was done to attempt to demonstrate such a relationship, especially with regard to in vivo product performance. Advances in instrumental methods and analytical precision ultimately opened up prospects for this work. The USP–NF Joint Panel on Physiologic Availability recognized that the disintegration test was insufficiently sensitive and in 1968 directed the identification of candidate articles for the first 12 official dissolution tests that used *Apparatus 1*.

USP requires drug release testing via the USP performance test in the majority of monographs for non-solution oral, sublingual, and transdermal dosage forms. In the current state of science, in vivo testing is necessary during the development and evaluation of both immediate-release and modified-release dosage forms. In some cases, depending on the Biopharmaceutics Classification System (BCS) classification of the drug, and depending on regulatory policy, in vivo testing may not be necessary. The special sensitivity of the dissolution test to changes in composition or method of manufacturing that do not result in significant changes in performance in vivo is well recognized. An understanding of the full complement of information given by in vitro and in vivo evaluation of the drug substance and product is the starting point in the development of a meaningful in vitro performance test.

IN VITRO EVALUATION

Physicochemical Properties—Drug Substance

Physicochemical information typically includes polymorphism, stability, particle size distribution, solubility, dissolution rate, lipophilicity, permeability, and other release-controlling variables of the drug substance under conditions that may mimic the extremes of the physiologic environment experienced by the dosage form.

Physicochemical Properties—Drug Product

The variables tested to characterize the physicochemical properties of the drug product should be the same as those that are tested to characterize the drug substance. Dissolution profiles over a relevant pH range, usually from pH 1–6.8, should be obtained with particular attention to formulation effects. Characterization of formulations that are insoluble in aqueous systems may require the addition of sodium lauryl sulfate or another surfactant. The BCS classification of the drug substance should be determined, especially for immediate-release dosage forms.

Dissolution Testing

Dissolution testing is required for all non-solution oral, including sublingual, Pharmacopeial dosage forms in which absorption of the drug is necessary for the product to exert the desired therapeutic effect. Exceptions include tablets that meet a requirement for completeness of solution, products that contain radiolabeled drugs, or products that contain a soluble drug and demonstrate rapid (10–15 min) disintegration. Dissolution testing should be conducted on equipment that conforms to the requirements in *Dissolution* ⟨711⟩ and on which a performance verification test has been conducted when one is available. On its website, USP provides a guidance for optimizing dissolution instrument performance by mechanical calibration and performance verification testing (https://www.usp.org/sites/default/files/usp/document/our-work/chemical-medicines/dissolution-toolkit-version2.pdf).

In vitro dissolution testing generally should attempt to mimic in vivo dissolution, but such in vitro conditions cannot be selected reliably a priori. A range of in vitro dissolution test conditions (e.g., media of varying pH, surfactant, and apparatus rotational speed) should be evaluated. Knowledge of drug substance properties, product formulation, gastrointestinal physiology, in vitro dissolution, and in vivo pharmacokinetics will aid in the selection of in vitro dissolution test conditions and specifications.

For products that contain more than a single active ingredient, dissolution typically should be determined for each active ingredient. When a dissolution test is added to an existing monograph, the disintegration test is deleted, but in the case of sublingual preparations and orally disintegrating tablets, disintegration may be a critical quality attribute in addition to dissolution. In such cases one or both tests can be included in the monograph.

When a single set of specifications cannot be established for multisource products described in monographs, multiple dissolution tests are allowed, and labeling is required to indicate the appropriate dissolution test for the specific product.

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Detailed information about method development and validation can be found in *The Dissolution Procedure: Development and Validation* ⟨1092⟩.

IMMEDIATE-RELEASE DOSAGE FORMS

For immediate-release dosage forms the in vitro dissolution process typically requires no more than 60 min, and in most cases a single time-point specification is adequate for Pharmacopeial purposes. To allow for typical disintegration times, test times of less than 30 min should be based on demonstrated need.

EXTENDED-RELEASE DOSAGE FORMS

For extended-release products in vivo dissolution generally is rate limiting, which results in protracted drug absorption and thus facilitates the identification of in vitro test conditions that may be predictive of in vivo dissolution. Multiple sampling time points, therefore, are necessary to define a dissolution profile for a modified-release dosage form.

The choice of apparatus should be based on knowledge of the formulation and actual dosage form performance in the in vitro test system. *Apparatus 1* (basket) or *Apparatus 2* (paddle) may be more useful at higher rotation rates (e.g., the paddle at 100 rpm). *Apparatus 3* (reciprocating cylinder) has been especially useful for bead-type modified-release dosage forms. *Apparatus 4* (flow cell) may offer advantages for modified-release dosage forms that contain active ingredients that have limited solubility. *Apparatus 7* (reciprocating disk) is applicable to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms. *Apparatus 5* (paddle over disk) and *Apparatus 6* (cylinder) also are useful for evaluating and testing transdermal dosage forms.

At least three timepoints are chosen to characterize the in vitro drug release profile of an extended-release dosage form for Pharmacopeial purposes. Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug.

IN VIVO EVALUATION OF DOSAGE FORMS

In evaluating a drug product's performance, analysts fundamentally must ask what type of study should be performed to give reasonable assurance of BE of a marketed product to the clinical trial product that demonstrated safety and efficacy. Although they provide important information concerning the release characteristics of the drug from the dosage form, in vitro dissolution studies at present are used primarily for setting or supporting specifications for drug products (e.g., shelf life) and manufacturing process control (e.g., scale-up or postapproval changes). Normally BE is best demonstrated by in vivo evaluation but can sometimes be replaced by in vitro studies.[1] BE assessment of modified-release dosage forms is best achieved by observing in vivo drug pharmacokinetic and/or pharmacodynamic behavior by means of well-designed clinical studies. Multiple guidances for the conduct of such studies are provided by regulatory agencies. Moreover, when a well-defined, predictive relationship exists between plasma concentrations of a drug or its active metabolites and the clinical response (therapeutic and adverse), it is possible to use plasma drug concentration data alone as a basis for the approval of a modified-release dosage form that is designed to replace an immediate-release dosage form.

Although human pharmacokinetic studies often are used to assess BE of immediate-release solid oral dosage forms, in some cases in vitro studies can be used to assess BE. The principal advantage of in vitro studies is that they reduce development costs. For example, an in vitro test is preferable when one is testing BCS Class I drugs with rapid dissolution. Some regulatory agencies permit this type of testing in lieu of in vivo testing.

The following discussions are intended to provide guidance for drug substance evaluation and the design, conduct, and evaluation of studies involving dosage forms. Although these guidelines focus on oral drug delivery systems, the principles may be applicable to other routes of drug administration (e.g., transdermal, subcutaneous, intramuscular, etc.).

CHARACTERIZATION OF DRUG SUBSTANCE

The Biopharmaceutics Classification System (BCS)

FDA has issued a guidance titled "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070246.pdf). A key assumption in the approach is that drug release and dissolution is sufficiently rapid so that an in vitro–in vivo correlation is not possible and/or useful. When applicable, the BCS allows dissolution rate data in lieu of BA or BE studies for product approval.

Pharmacokinetic Properties

Analysts should thoroughly characterize the input absorption profile of the active drug entity from a formulation that shows rapid BA (an intravenous solution, oral solution, or a well-characterized immediate-release drug product). In turn, this formulation serves as a reference to evaluate the input profile of the modified-release dosage form. This information, together with the pharmacokinetics of the active drug entity, can characterize drug absorption and can predict changes in drug BA when input is modified as in modified-release dosage forms. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism, a reduction in systemic availability could result after oral administration if the input rate is decreased.

[1] 21 CFR 320.22 Criteria for waiver of evidence of in vivo bioavailability or bioequivalence.

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In designing an oral modified-release dosage form, analysts may find it useful to determine the absorption of the active drug entity in various segments of the gastrointestinal tract, particularly in the lower gastrointestinal tract (colon) for delayed-release dosage forms that release drug in this region. Food effects also may be important and should be investigated.

Drug Disposition

The information required to characterize drug disposition may include the following.

1. Disposition parameters—clearance, area under the time—plasma concentration curve (AUC), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), volume of distribution, half-life, mean residence time, or model-dependent parameters.
2. Linearity or characterization of nonlinearity over the dose or concentration range that could be encountered.
3. Drug/metabolite accumulation.
4. Metabolic profile and excretory pathway, with special attention to the active metabolites and active enantiomers of racemic mixtures.
5. Enterohepatic circulation.
6. Protein-binding parameters and effect of dialysis.
7. The effects of age, gender, race, and relevant disease states.
8. Plasma: blood ratios.
9. A narrow therapeutic index or a clinical response that varies significantly as a function of the time of day (chronopharmacokinetics).

Pharmacodynamic Properties

Before developing a dosage form, analysts should obtain concentration–response relationships over a dose range sufficiently wide to encompass important therapeutic and adverse responses. In addition, the equilibration-time[2] characteristics between plasma concentration and effect should be evaluated. For modified-release products that typically have larger drug doses in the dosage form, these concentration–response relationships should be sufficiently characterized so that a reasonable prediction of the safety margin can be made if dose dumping should occur. If there is a well-defined relationship between the plasma concentration of the active drug substance or active metabolites and the clinical response (therapeutic and adverse), the clinical performance of a new modified-release dosage form could be characterized by plasma concentration–time data. If such data are not available, clinical trials of the modified-release dosage form should be carried out with concurrent pharmacokinetic and pharmacodynamic measurements.

CHARACTERIZATION OF THE DOSAGE FORM

Pharmacokinetic Properties: Immediate-Release Products

The types of pharmacokinetic studies that should be conducted are based on how much is known about the active drug substance, its clinical pharmacokinetics, and its BCS Class. For example, a new chemical entity requires greater pharmacokinetic characterization than does an FDA-approved formulation that is undergoing scale-up and postapproval changes (SUPAC) evaluation.

The latter is seen when an FDA-approved drug product undergoes changes in the manufacturing of the product after the product has been approved. Such changes are common and can be caused by expansion in the size of the lots manufactured, new manufacturing locations, or the introduction of new technology. Necessary in vitro dissolution tests and/or in vivo BE tests are described in the FDA "Guidance for Industry: Immediate-release Solid Oral Dosage Forms: Scale-up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070636.pdf).

Similar requirements apply to a generic equivalent of an approved immediate-release dosage form that must be BE to the innovator drug, known as the reference listed drug. The two most frequently used methods for meeting bioequivalence requirements are in vivo pharmacokinetic studies and BCS-based in vitro studies.

Pharmacokinetic Properties: Modified-Release Products

Like the approaches for immediate-release products, the types of pharmacokinetic studies that should be conducted for modified-release products are based on how much is known about the drug substance, its pharmacokinetics, biopharmaceutics, and whether pharmacokinetic studies are intended to be the sole basis for product approval. At a minimum, two studies are required to characterize the product when no reference modified-release product exists: (1) a single-dose crossover study for each strength of a modified-release dosage form and (2) a multiple-dose, steady-state study using the highest strength of a modified-release dosage form. A food effects study to evaluate the potential for dose dumping from extended-release dosage forms also is required as a separate study or is included as an arm of a crossover study. In the demonstration of interchangeability,

[2] Equilibration time is a measure of the time-dependent discontinuity between measured plasma concentrations and measured effects. The discontinuity is more often characterized by the degree of hysteresis observed when the effect-concentration plot for increasing concentrations is compared with that for decreasing concentrations. Where the equilibration time is very short (i.e., rapid equilibration with no active metabolites generated), there will be little or no hysteresis. That is, the same effect will be observed for a given concentration independent of the interval between the time of dosing and the time that measurements are made.

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a single-dose, fasting crossover study vs. the reference product usually will suffice. In some cases, a food-effects study is required if the reference product has demonstrated a food effect on BA. Some appropriate single-dose crossover and multiple-dose steady-state studies are described below.

For modified-release products, intravenous solutions, oral solutions, or well-characterized immediate-release drug products are possible reference products to evaluate a modified-release formulation. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism from the small intestine, a reduction in systemic availability could result after oral administration if the input rate is decreased. An increase in systemic availability could be observed if a drug is absorbed from the colon from a delayed-release dosage form that targets the colon, thus avoiding a first-pass effect.

In some modified-release capsule dosage forms, the strengths differ from each other only in the amount of identical beaded material contained in each capsule. In this case, single-dose and multiple-dose steady-state studies at the highest dosage strength are sufficient. Other strengths can be characterized on the basis of comparative in vitro dissolution data.

The pharmacokinetic studies described below are needed for most modified-release dosage forms. These studies may be the basis for characterization of the dosage form. If regulatory approval is sought without conducting clinical trials, manufacturers should consult with the regulatory authorities to ensure that an adequate database exists for the approval. The types of pharmacokinetic studies generally conducted can be categorized as follows.

CASE A

Case A applies to an original modified-release oral dosage form for a drug already marketed in an immediate-release dosage form and for which extensive pharmacokinetic/pharmacodynamic data exist.

Single-dose crossover study: A single-dose crossover study should include the following treatments: the modified-release dosage form administered under fasting conditions; a dosage form that is rapidly available administered under fasting conditions; and the modified-release dosage form administered immediately after a high-fat standardized meal. The food effects study should control the ambient-temperature fluid intake (e.g., 6–8 oz.) at the time of drug administration. The dosage form should be administered within 5 min after completion of the meal. Ideally all subjects should consume the meal in approximately 15 min. If there are no significant differences in the rate or extent of bioavailability (AUC, C_{max}, and T_{max}) as a function of the meal, then additional food effect studies are not necessary. If significant differences in bioavailability are found, researchers must define how food affects the modified-release dosage form,[3] as well as how the food–drug effect relates to time.

Use the following guidelines in evaluating food effects.

1. If no well-controlled studies have previously defined the effects of a concurrent high-fat meal on an immediate-release dosage form, studies should be performed to determine whether a food effect is a result of problems with the dosage form. Does the dosage form show food-related changes in release, or are there food effects that are unrelated to the dosage form, e.g., changes in the drug's absorption from the gastrointestinal tract or changes in the drug's disposition that are independent of absorption? The cause of the food effect should be determined by a single-dose crossover study comparing the solution (or immediate-release dosage form) under fed and fasting conditions. If there is no food effect, then one concludes that there are problems with the dosage form. If there is a food effect, then one concludes that the effect is unrelated to the dosage form.
2. The influence of timing on the food effect should be tested by a four-way crossover study, in which the modified-release dosage form is administered under the following treatment conditions: fasting, taken with a high-fat meal, 1 h before a high-fat meal, and 2 h after a high-fat meal.
3. If the food effect on an immediate-release dosage form is determined to result from changes in the dissolved drug's absorption from the gastrointestinal tract or from changes in drug disposition, studies should define the appropriate relationship between drug dosing and meals.
4. Alternative appropriate studies can be conducted if the applicant labels the drug for administration with a meal that is not fat loaded. In this case, an alternative meal composition should be considered.
5. Analysts should monitor the entire single-dose, modified-release absorption profile. Where appropriate (e.g., in a multiple-dose study) for specific drugs and drug delivery systems, blood samples should be taken following breakfast on the second day, before the second dose is administered. This sampling schedule is particularly important for once-a-day products.
6. For delayed-release (enteric-coated) dosage forms, analysts should perform BA studies to characterize food effect and to support the dosing claims stated in the labeling.

The purpose of these studies is twofold: first, to determine whether a need exists for labeling instructions describing special conditions for administration with respect to meals; and second, to provide information concerning the pattern of absorption of the modified-release dosage form compared to that of the immediate-release dosage form. Drug input function should be defined for modified-release dosage forms. This will aid in the development of an appropriate in vitro dissolution test. For dosage forms that exhibit high variability, a replicate study design is recommended.

Multiple-dose, steady-state studies

Study I—When data demonstrating linear pharmacokinetics exist for an immediate-release dosage form, a steady-state study should be conducted with the modified-release dosage form at one dose rate (preferably at the high end of the usual dosage regimen) using a comparable total daily dose of an immediate-release dosage form as a control. At least three trough plasma drug concentration (C_{min}) determinations at the same time of day should be made to demonstrate that steady-state conditions have been achieved. Plasma drug concentration determinations, over at least one dosing interval of the modified-release dosage form, should be made in each phase of the crossover study. It may be preferable (as in the case of rhythmic variation in absorption or disposition of the drug) to measure concentrations over an entire day in each phase. The presence or absence of circadian variation should be verified. The modified-release dosage form should produce an AUC that is equivalent to that of the immediate-release dosage form if the extent of absorption from the modified-release dosage form is comparable to the

[3] Wagner–Nelson, Loo–Riegelman, and other deconvolution methods are found in textbooks on biopharmaceutics.

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immediate-release dose. The degree of fluctuation for the modified-release product should be the same as, or less than, that for the immediate-release dosage form given by the approved regimen. Appropriate concentration measurements should include unchanged drug and major active metabolites. For racemic drug entities, analysts should consider measurement of the active enantiomers.

Study II—When comparisons of the pharmacokinetic properties of an immediate-release dosage form at different doses are not available, or when the data demonstrate nonlinearity, steady-state crossover studies comparing effects of the modified-release dosage form and those of the immediate-release dosage form should be conducted at two different dose rates: one at the low end of the recommended dosing range and the second at the high end of the dosing range. In each case, the modified-release dosage form must meet the criteria described in *Study I* with respect to AUC and fluctuations in plasma drug concentrations. If there are significant differences between the modified-release dosage form and the immediate-release dosage form at either the low or the high dosing rate, these data alone are not adequate to characterize the product. Data can be misleading when obtained from subjects with atypical drug disposition or physiologic characteristics relative to the target population. Therefore, subject selection should be from an appropriate target population with randomized assignment to dosage form population. If the modified-release dosage form is for use in a specific subpopulation (e.g., for children), it should be tested in that population. Whether a drug exhibits linear or nonlinear pharmacokinetics, the basis for characterization is equivalence of AUC and of the relative degree of fluctuation of concentrations of the modified-release and immediate-release dosage forms.

Steady-state studies in selected patient populations or drug interaction studies may also be necessary, depending on the therapeutic use of the drug and the types of individuals for whom the modified-release dosage form will be recommended. For drugs that have narrow therapeutic indices, it may be necessary to perform more extensive plasma concentration measurements to determine the potential for unusual drug-release patterns in certain subpopulations. In such studies, researchers should perform more than one AUC measurement per patient to assess variability with both the modified-release and the immediate-release dosage forms.

CASE B

Case B applies to a non-oral, modified-release dosage form of an already marketed active drug entity for which extensive pharmacokinetic and pharmacodynamic data exist.

Case A studies (omitting the food effects studies) are appropriate for the evaluation of a modified-release dosage form designed for a non-oral route of administration if the pattern of biotransformation to active metabolites is identical for the two routes. If the biotransformation patterns are different, then clinical efficacy studies should be performed with the modified-release dosage form. In addition, special studies may be necessary to assess specific risk factors related to the dosage form (e.g., irritation and/or sensitization at the site of application of a transdermal drug delivery system).

CASE C

Case C applies to a generic equivalent of an approved modified-release dosage form, which should be BE to the reference drug in its rate and extent of drug exposure (i.e., AUC, C_{max}, C_{min}, and degree of fluctuation) in crossover single-dose studies. For an oral modified-release dosage form, the food studies described under *Case A* also should be performed.

CASE D

Case D applies to an FDA-approved product that has undergone SUPAC. Necessary in vitro dissolution tests and/or in vivo bioequivalence tests are described in the FDA guidance, *SUPAC-MR: Modified Release Solid Oral Dosage Forms; Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation* (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070640.pdf).

Statistical Analysis of In Vivo Bioequivalence

An appropriate statistical method should be selected. (See *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩).

IN VITRO–IN VIVO CORRELATIONS

The term IVIVC first appeared in the pharmaceutical literature as a result of the awareness of the importance of bioavailability concepts and in vitro dissolution rate determinations. IVIVC refers to the establishment of a rational relationship between a biological property, or a parameter derived from drug plasma concentrations produced by a dosage form, and a physicochemical property or characteristic of the same dosage form. The biological properties most commonly used are one or more pharmacokinetic parameters such as C_{max} or AUC, obtained following the administration of the dosage form. The physicochemical property most commonly used is a dosage form's in vitro dissolution behavior (e.g., percent of drug released under a given set of conditions). The quantitative relationship between the two properties, biological and physicochemical, is an IVIVC. The most important use of an IVIVC is for predictability. In many cases the actual drug plasma concentration profile can be predicted from in vitro dissolution data.

Historically, IVIVC analysis has been more successful for extended-release products than for immediate-release products. This difference probably reflects the application of specific data analysis techniques and interpretations that require dissolution rate–limited drug absorption. However some correlations with immediate-release products have been demonstrated using methods that rely on the current, broad availability of computers and nonlinear regression software, along with new correlation methods.

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General Considerations

With the proliferation of modified-release products, it becomes necessary to examine IVIVC in greater detail. Unlike immediate-release dosage forms, modified-release products, particularly extended-release dosage forms, cannot be characterized using a single time point dissolution test. These products are designed to deliver drug so that a patient has a specific plasma level profile over a prolonged period, usually 12–24 h. Analysts require an in vitro means of ensuring that each batch of the product will perform identically in vivo. An IVIVC satisfies this requirement. Initially, it was thought that developing a meaningful correlation for immediate-release dosage forms would be an easier task than for extended-release products. However, because of the nature of the principles on which each type is based, analysts now believe that an IVIVC is more readily achieved for modified-release dosage forms.

One expects all extended-release products to be dissolution rate limited. For these products, the formulation significantly contributes to the prolongation of drug release from the dosage form. Because of the impact of formulation on BA from an extended-release product, numerous attempts have been made to correlate one or more pharmacokinetic parameters determined from in vivo studies with the amount released in a given time during an in vitro dissolution test. Single-point correlations can indicate that increasing or decreasing the in vitro dissolution rate of the modified-release dosage form would result in a corresponding directional change in the product's performance. However, such single-point correlations reveal little about the overall plasma level curve, which is a major factor for drug performance in the patient. Rather, correlation methods that utilize all plasma drug concentration data and all in vitro dissolution data are preferred. Three correlation procedures are available that use all dissolution and plasma data, along with statistical moment analysis. Each procedure displays important differences in the quality of the correlation. These methods are discussed in terms of the advantages of each along with its potential utility as a predictive tool for pharmaceutical scientists.

Correlation Levels

Three correlation levels have been defined and categorized in descending order of quality. The concept of correlation level is based on the ability of the correlation to reflect the entire plasma drug concentration–time curve that results from administration of the given dosage form. The relationship of the entire in vitro dissolution curve to the entire plasma concentration–time profile defines the strength of the correlation and, therefore, the predictability.

LEVEL A

This level is the highest category of correlation. It represents a point-to-point relationship between in vitro dissolution and the in vivo input rate (absorption rate of the drug from the dosage form). For a *Level A* correlation, a product's in vitro dissolution curve is compared to its in vivo input curve, i.e., the curve produced by deconvolution of the plasma profile. Deconvolution can be accomplished using mass balance model-dependent methods, such as the Wagner–Nelson or Loo–Riegelman methods, or by model-independent, mathematical deconvolution. In an ideal correlation, the in vitro dissolution and in vivo absorption rate curves are superimposable or can be made superimposed by the use of a constant offset value of the time scale. The equations describing each curve are the same. This procedure often is found with modified-release dosage systems that demonstrate an in vitro release rate that is essentially independent of the dissolution media and stirring speeds used in a dissolution apparatus. Superimposition is not an absolute requirement for a *Level A* correlation. If the dissolution and absorption curves are different and a mathematical relationship can be developed to relate the two, the plasma level profile still is predictable from the in vitro dissolution data. This relationship must be true not only at that single input rate but also over the entire quality control dissolution range for the product. Furthermore, when the dissolution rate depends on mixing speed, the two curves can be made to superimpose by either increasing or decreasing the in vitro mixing speed or some other alteration of the dissolution method.

The advantages of a *Level A* correlation are as follows.

1. It develops a point-to-point correlation. This is not found with any other correlation level. It is developed using every plasma level and dissolution point collected at different time intervals, so it reflects the complete plasma level curve. As a result, in the case of a *Level A* correlation an in vitro dissolution curve can serve as a surrogate for in vivo performance. A change in manufacturing site, method of manufacture, raw material supplies, minor formulation modifications, and even product strength using the same formulation can be justified without the need for additional BA-BE studies.[4,5]
2. A truly meaningful quality control procedure that indicates in vivo performance and is predictive of a dosage form's performance is defined for the dosage form.
3. The extremes of the in vitro quality control standards can be justified either by convolution (simulating the plasma level profile from the dissolution curve) or by deconvolution (using the upper and lower confidence interval limits).

LEVEL B

This correlation uses the principles of statistical moment analysis. The mean in vitro dissolution time is compared to either the mean residence time or the mean in vivo dissolution time. As with a *Level A* correlation, *Level B* uses all of the in vitro and in vivo data but is not considered a point-to-point correlation. It does not correlate the actual in vivo plasma profiles but rather a parameter that results from statistical moment analysis of a plasma profile component such as mean residence time. Because a

[4] FDA Guidance SUPAC-MR: Modified Release Solid Oral Dosage Forms—Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation (1997).

[5] FDA Guidance Extended-Release Solid Oral Dosage Form—Development, Evaluation, and Application of In Vitro/In Vivo Correlations, "If an IVIVC is developed with the highest strength, waivers for changes made on the highest strength and any lower strengths may be granted if these strengths are compositionally proportional or qualitatively the same, the in vitro dissolution profiles of all the strengths are similar, and all strengths have the same release mechanism."

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number of different plasma profiles can produce similar mean residence time values, one cannot rely on a *Level B* correlation alone to predict a plasma profile from in vitro dissolution data. In addition, in vitro data from such a correlation cannot be used to justify values at the extremes of quality control standards.

LEVEL C

This category relates one dissolution time point ($t_{50\%}$, $t_{90\%}$, etc.) to one pharmacokinetic parameter such as AUC, C_{max}, or T_{max}. It represents a single-point correlation and does not reflect the complete shape of the plasma profile, which best defines the performance of modified-release products. Because this type of correlation is not predictive of actual in vivo product performance, generally it is useful only as a guide in formulation development or as a production quality control procedure. Because of its obvious limitations, a *Level C* correlation has limited usefulness in predicting in vivo drug performance and is subject to the same caveats as a *Level B* correlation in its ability to support product and site changes as well as justification of the extreme values in quality control standards. The FDA Guidance "Extended-Release Solid Oral Dosage Forms—Development, Evaluation, and Application of In Vitro/In Vivo Correlations" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070239.pdf) states that manufacturers can obtain biowaivers based on multiple *Level C* correlations. The guidance shows how manufacturers can achieve this correlation. The FDA also indicates that if such a correlation is achievable, it is likely that the development of a *Level A* correlation is also feasible.

DEVELOPING A CORRELATION

This chapter does not define the only procedures for developing an IVIVC, and any well-designed and scientifically valid approach is acceptable. To assist the pharmaceutical scientist, one possible procedure for developing a *Level A* correlation is described below:

1. In order to perform deconvolution properly, analysts should be familiar with the pharmacokinetics of the drug itself as well as when it is incorporated into a modified-release dosage form. For example, if a drug is known to be fully absorbed but demonstrates saturable first-pass kinetics, it is best to assume 100% bioavailability for purposes of absorption rate calculation. This is based upon the fact that the drug is fully absorbed, but because of liver metabolism, one sees less than if the drug were administered as an immediate-release bolus. If one utilizes the extent of absorption relative to an immediate-release or solution dosage form, the input profiles will not superimpose with that calculated assuming 100% absorption. However, point-to-point correlations most likely will be possible.
2. Different dissolution profiles of a formulation should be obtained as illustrated in *Figure 1*. The formulation should be modified only sufficiently to produce different dissolution profiles so that the formulation has the same excipients in all the lots that will be tested. The formulation modifications used in these batches should be based on factors that would be expected to influence the product's modified-release rate and could occur during normal product manufacture. In vitro drug release is performed on the batches that will be used in the bioavailability study, and the effect of varying the dissolution conditions is investigated. Some of the variables that should be studied are the apparatus (it is preferable to use official dissolution equipment), mixing intensity, and dissolution medium (i.e., pH value, enzymes, surfactants, osmotic pressure, ionic strength, etc.). The dissolution behavior of the dosage form need not be studied under all of the conditions indicated. The number of conditions investigated depends largely on whether a correlation can be developed with the in vitro results obtained under the more commonly investigated conditions such as apparatus, agitation intensity, or dissolution medium and pH value. Each formulation and every drug represents an individual challenge. The resulting dissolution profiles from the use of different dissolution media are illustrated in *Figures 1* and *2* in which the same formulations were tested in water and an acid buffer.

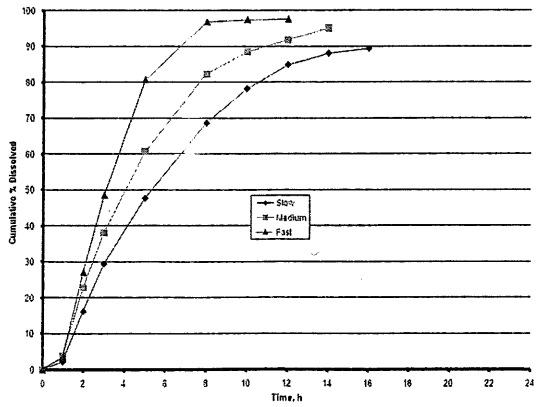

Figure 1. Mean dissolution profiles of three modifications of a new modified-release formulation (USP Apparatus 2, 50 rpm, 0.9 L water, 37°).

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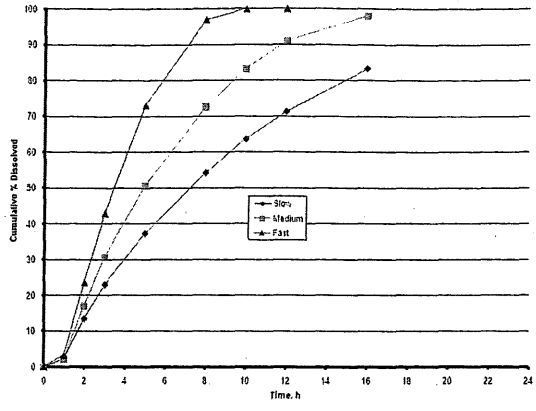

Figure 2. Mean dissolution profiles of a new modified-release formulation (USP Apparatus 2, 50 rpm, 0.9 L, pH 4.5 buffer, 37°).

3. The plasma level or urinary excretion data obtained in the definitive bioavailability study of the modified-release dosage form are treated by a deconvolution procedure. The resulting data may represent the drug input rate of the dosage form. They also represent in vivo dissolution when the rate-controlling step of the dosage form is its dissolution rate (i.e., drug absorption after dissolution is considered to be instantaneous). Any deconvolution procedure (e.g., mass balance or mathematical deconvolution) will produce acceptable results. *Figure 3* illustrates the results of numerical deconvolution of the plasma profiles obtained for the batches in *Figures 1* and *2*.

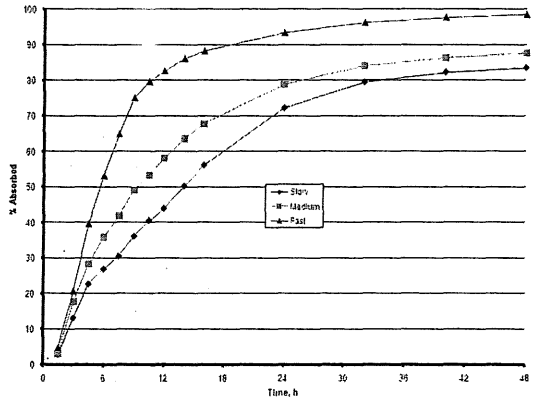

Figure 3. Mean absorption profiles from numerical deconvolution of plasma concentration–time plots.

4. The in vitro dissolution curve is then compared to the drug absorption rate curve. This can be performed by various methods. Simply positioning one curve on the other often can indicate the existence of a correlation. This may then be quantified by defining the equation for each curve and comparing the corresponding constants. The simplest way to demonstrate a correlation is to plot the fraction absorbed in vivo vs. the fraction released in vitro, as illustrated in *Figures 4* and *5*. With a *Level A* correlation, this relationship is often linear with a slope approaching 1. As illustrated in *Figures 4* and *5*, a correlation may be curvilinear. The intercept may or may not be zero depending upon whether there is a lag time before the system begins to release drug in vivo, or the absorption rate is not instantaneous, resulting in the presence of some finite quantity of dissolved but unabsorbed drug. In either case, it is a point-to-point or a *Level A* correlation when the least-squares fit of the line approaches a coefficient of determination, R^2, of 1. For the correlations illustrated in *Figures 4* and *5*, the IVIVC using the acid buffer dissolution profiles was superior to that obtained from water.

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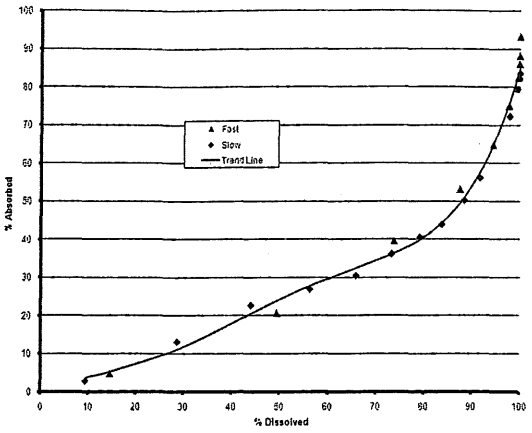

Figure 4. IVIVC attempt: water (using slow and fast formulations).

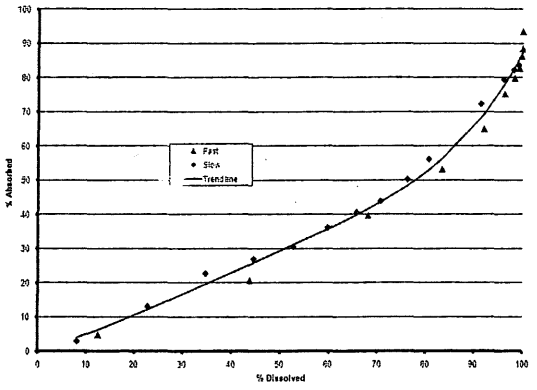

Figure 5. IVIVC attempt: pH 4.5 buffer.

5. If from the studies indicated in the in vitro dissolution evaluation, given above, the modified-release dosage form exhibits dissolution behavior that is independent of the variables studied and a *Level A* correlation is demonstrated when the in vitro dissolution curve is compared to the drug input rate curve, then it is likely that the correlation is general and can be extrapolated within a reasonable range for that formulation of the active drug substance. If the dosage form exhibits dissolution behavior that varies with the in vitro conditions, analysts must determine which set of dissolution conditions best correlates with in vivo performance. One can then establish whether the correlation is real or an artifact. This is achieved by preparing at least two formulations that have significantly different in vitro behavior. One should demonstrate a more rapid release and the other a slower release than the clinical bioavailability lot (biobatch). A pilot BA-BE study should be performed with these formulations, and the previously established correlation should be demonstrated for both. The formulation modifications of these batches should be based upon formulation factors that would be expected to influence the product's modified-release mechanism, and modification of these formulation factors are expected to influence the dosage form's release rate.
6. Alternatively, the in vivo performance of the biobatch formulation can be simulated based on the correlation developed with these formulations that were used in the BA-BE study. Analysts then can compare the predicted and experimentally determined values, the prediction error. The exercise illustrated in *Figures 6* and *7* serves as an internal validation of the *Level A* correlation. An external validation would involve simulating data for a formulation batch that was not included in the *Level A* correlation calculations. Such a validation was performed using the in vivo data from the medium lot of the formulation, and the results are illustrated in *Figure 8*.

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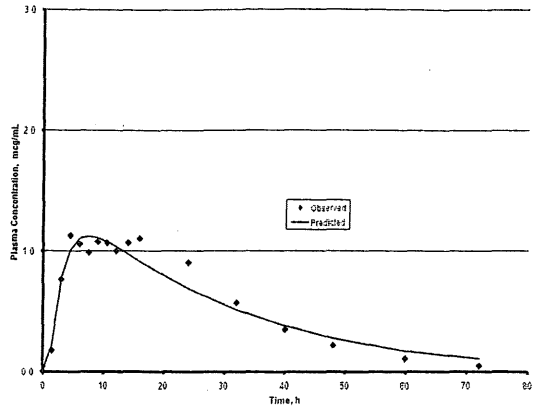

Figure 6. Observed and predicted mean plasma profiles: slow formulation.

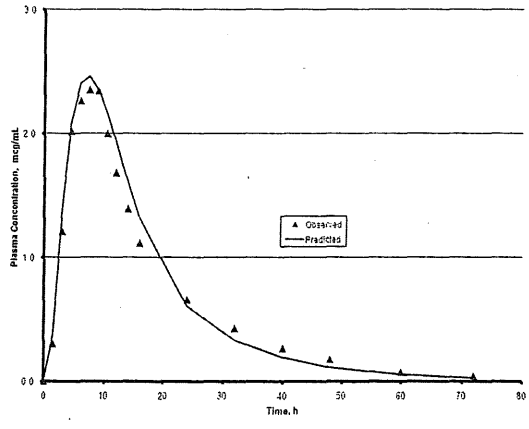

Figure 7. Observed and predicted mean plasma profiles: fast formulation.

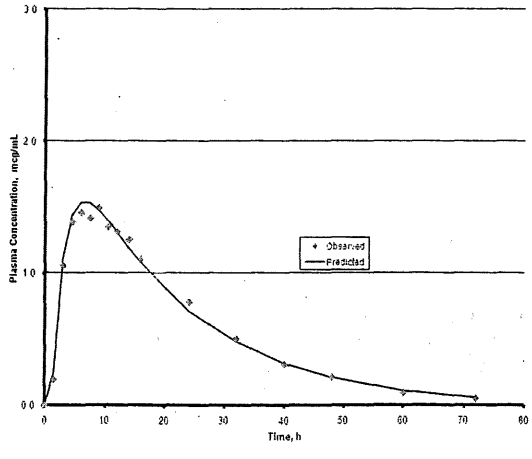

Figure 8. Observed and predicted mean plasma profiles: medium formulation.

7. Once a *Level A* correlation is established, in vitro testing can be used to establish dissolution specifications, biowaivers to facilitate SUPAC, and changes in dosage form strength for the same formulation. It is questionable whether such an extrapolation with *Level B* and *C* correlations is possible.

Establishment of Dissolution Specification Ranges

It is relatively easy to establish a multipoint dissolution specification for a modified-release dosage form. The dissolution behavior of the biobatch can be used to define the amount that will be released at each time point. The difficulty arises in the

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variation that will be allowed around each time point. In the case of a *Level A* correlation, this can be done in two ways, both of which use IVIVC: convolution and deconvolution.

CONVOLUTION

Reasonable upper and lower dissolution values are selected for each time point established from the biobatch. Historically, dissolution specifications have been selected by using the average dissolution of the development batches, with a range of ±2.5–3 standard deviations. It is now expected that the average dissolution values be approximately the same as those of the biobatch. The dissolution curves defined by the upper and lower extremes are convoluted to project the anticipated plasma level curves that would result from administration of these formulations to the same patients to whom the biobatch was administered. If the resulting plasma level data fall within the 95% confidence intervals obtained in the definitive BA-BE study, these ranges can be considered acceptable. An alternative acceptance approach that can be used after the therapeutic window for a drug has been defined, is to establish whether the upper and lower limits of the convolution results fall within the therapeutic window, even if they fall outside the confidence interval. If they fall outside the window, a more limited range must be established. This procedure should be continued until the predicted values meet the desired ranges.

DECONVOLUTION

An acceptable set of plasma-level data is established both for a batch of material demonstrating a more rapid release and for one demonstrating a slower release than that of the biobatch. These can be selected by using the extremes of the 95% confidence intervals or ±1 standard deviation of the mean plasma level. These curves are then deconvoluted, and the resulting input rate curve is used to establish the upper and lower dissolution specifications at each time point. In the case of *Level B* and *C* correlations, batches of product must be made at the proposed upper and lower limits of the dissolution range, and it must be demonstrated that these batches are acceptable by a BA-BE study.

Immediate-Release Dosage Forms

GENERAL CONSIDERATIONS

Because the mechanisms for drug release from modified-release dosage forms are more complex and variable than those associated with immediate-release dosage forms, one would anticipate that an IVIVC would be easier to develop with the latter formulations. Unfortunately, most of the correlation efforts to date with immediate-release dosage forms have been based on the correlation *Level C* approach, although there also have been efforts employing statistical moment theory (*Level B*). Although it is conceivable that the same *Level A* correlation approach can be used with immediate-release dosage forms, until data have been gathered to support this concept, *Level B* and *Level C* are the best approaches that can be recommended with these dosage forms.

Change to read:

⟨1090⟩ ASSESSMENT OF ▲SOLID ORAL▲ (USP 1-May-2019) DRUG PRODUCT PERFORMANCE ▲AND INTERCHANGEABILITY,▲ (USP 1-May-2019) BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

Change to read:

BACKGROUND
BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION
BIOEQUIVALENCE
- Immediate-Release Drug Products
- Modified-Release Drug Products
- Orally Administered Drug Products, Not for Systemic Effect
- Bioequivalence Studies
- Statistical Analysis

DISSOLUTION AND IN VITRO PRODUCT PERFORMANCE
- Dissolution and In Vitro BA
- Dissolution and In Vitro Equivalence
- Dissolution Profile Comparisons

▲INTERCHANGEABILITY OF DRUG PRODUCTS
SOLID FORM AND PARTICLE SIZE
DIFFERENCES IN EXCIPIENTS
MANUFACTURING PROCESS▲ (USP 1-May-2019)
BIOWAIVER

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Biowaiver Based on Dosage Form Proportionality
Biowaiver Based on the BCS
APPENDIX

Change to read:

BACKGROUND

This chapter provides recommendations for the in vivo and in vitro assessment of ▲solid oral▲ (USP 1-May-2019) drug product performance. The chapter is intended as a guide for scientists and clinicians seeking to evaluate drug product performance by surrogate procedures correlative and/or antecedent to clinical trials in humans. *USP–NF* provides quality standards for drug substances, excipients, and finished preparations. A *USP–NF* monograph for an official substance or preparation includes the article's definition; packaging, storage, and other requirements; and a specification. The specification consists of a series of universal tests (description, identification, impurities, and assay) and specific tests—one or more analytical procedures for each test—and acceptance criteria. Quality standards are important attributes that must be built into the drug product. Meeting *USP–NF* standards is accepted globally as assurance of high quality and is part of the requirements necessary for approval of a bioequivalent▲ ▲ (USP 1-May-2019), interchangeable ▲ ▲ (USP 1-May-2019) drug product. ▲ ▲ (USP 1-May-2019) Drug products must meet certain in vivo and/or in vitro performance standards to be considered therapeutically equivalent (TE) and interchangeable. ▲ ▲ (USP 1-May-2019) Drug product performance may be defined as the release of the ▲drug substance▲ (USP 1-May-2019) from the drug product dosage form, ▲normally▲ (USP 1-May-2019) leading to systemic ▲exposure or less often to local activity▲ (USP 1-May-2019) of the ▲drug substance▲ (USP 1-May-2019) necessary for achieving a desired therapeutic response. ▲Bioavailability (BA) is a measure of systemic exposure.▲ (USP 1-May-2019) This chapter discusses in vivo and in vitro approaches to determining drug product performance. The focus of the chapter is primarily on the performance of solid oral drug products.

▲This▲ (USP 1-May-2019) chapter references ▲two▲ (USP 1-May-2019) Food and Drug Administration (FDA) ▲guidances: *Guidance for Industry: Bioavailability and Bioequivalence Studies Submitted in NDAs or INDs—General Considerations* (draft guidance March 2014) and *Guidance for Industry: Bioequivalence Studies with Pharmacokinetic Endpoints for Drugs Submitted Under an ANDA* (draft guidance December 2013) (search by document title; http://www.fda.gov/); a European Medicines Agency (EMA) guidance: *Guideline on the Investigation of Bioequivalence* (2010) (search by document title; http://www.ema.europa.eu/ema/),▲ (USP 1-May-2019) and a World Health Organization (WHO) document, *Annex 7: Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability*, ▲WHO Expert Committee on Specifications for Pharmaceutical Products: Fortieth Report. World Health Organization: Geneva; 2006: Annex 7 (WHO Technical Report Series, No. 937). TRS 992 (2015)▲ (USP 1-May-2019) (search by document title; http://who.int/en/). FDA guidances are used in the United States, and WHO, FDA, ▲EMA,▲ (USP 1-May-2019) and national/regional guidelines may be used by national/regional drug regulatory authorities ▲outside the U.S.▲ (USP 1-May-2019) Following approval, control of the quality of a drug product can be achieved in part by using the private and/or public specification, which can include a performance test. USP provides the general chapters *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩, ▲ ▲ (USP 1-May-2019) *The Dissolution Procedure: Development and Validation* ⟨1092⟩, and *Capsules—Dissolution Testing and Related Quality Attributes* ⟨1094⟩, which describe these tests and procedures.

This chapter provides general information about the conduct of bioequivalence (BE) studies as a surrogate measure of in vivo drug product performance and dissolution profile comparisons as a measure of in vitro drug product performance ▲as stated in the guidelines cited in this chapter.▲ (USP 1-May-2019) The chapter also discusses conditions when an in vivo BE requirement may be waived (biowaiver) for certain drug products and shows how the Biopharmaceutics Classification System (BCS) can be used as a predictor of a drug product's performance. An *Appendix* to this chapter defines key scientific terminology and provides a comparison between FDA, ▲EMA,▲ (USP 1-May-2019) and WHO in terms of drug product performance assessment. ▲Other BE guidance may exist outside of that given in this chapter.▲ (USP 1-May-2019)

Change to read:

BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

BA studies focus on determining the process and time frame by which a drug ▲substance▲ (USP 1-May-2019) is released from the oral dosage form and moves to the site of action [see ▲*Guidance for Industry: Bioavailability and Bioequivalence Studies Submitted in NDAs or INDs—General Considerations* (FDA draft guidance March 2014) and *Guidance for Industry: Bioequivalence Studies with Pharmacokinetic Endpoints for Drugs Submitted Under an ANDA* (FDA draft guidance December 2013) (search by document title; http://www.fda.gov/].▲ (USP 1-May-2019) BA is an indirect or surrogate measure of the rate and extent to which the ▲drug substance▲ (USP 1-May-2019) or active moiety is absorbed from a drug product and becomes available at its target sites of action. BA data provide an estimate of systemic drug exposure, including fraction of drug absorbed. ▲The determination of availability of drug substance from drug products that are not intended to be absorbed into the bloodstream is outside the scope of this chapter.▲ (USP 1-May-2019) Drug products are considered bioequivalent if a test ▲(*T*)▲ (USP 1-May-2019) drug product does not show a significant difference in rate and extent of absorption by comparison with a designated reference ▲(*R*) drug product▲ (USP 1-May-2019) when administered at the same ▲ ▲ (USP 1-May-2019) dose ▲ ▲ (USP 1-May-2019) under similar experimental conditions in either a single dose or in multiple doses.

BA and BE generally can be obtained by serially measuring drug ▲substance▲ (USP 1-May-2019) and/or metabolite concentrations in the systemic circulation over a prescribed period. BE studies can use other approaches when systemic drug concentrations cannot be measured or are not appropriate. For these cases, more indirect approaches to BE determination include acute

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pharmacodynamic endpoints, clinical endpoints, and in vitro studies that typically involve comparisons of the dissolution profiles of ▲(*T*)▲ (USP 1-May-2019) and ▲(*R*)▲ (USP 1-May-2019) drug products. ▲In the absence of an in vitro–in vivo correlation (IVIVC) or considerations based on the BCS, in vitro performance studies are not used in place of BA studies.▲ (USP 1-May-2019)

BA and BE information are important in regulatory submissions. BA information broadly addresses the absorption, distribution, metabolism, and excretion of the ▲drug substance.▲ (USP 1-May-2019) For an innovator product, BE studies establish the performance of the product intended for marketing by comparing the BA of the product as developed for marketing approval to the clinical trial material, the drug product used in safety/efficacy trials. For the development and regulatory approval of a generic drug product, the ▲*T*▲ (USP 1-May-2019) must be ▲bioequivalent▲ (USP 1-May-2019) to the ▲*R*▲ (USP 1-May-2019) (usually the brand or innovator drug product that is designated by the applicable regulatory authority).

The ICH guideline document titled *Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances Q6A* (2000) (search by document title; http://www.fda.gov/) provides approaches for setting acceptance criteria for drug product performance. These approaches rely on dissolution or disintegration based on clinically acceptable batches, as does FDA's approach. BE studies focus on the performance of the drug product and usually involve comparisons of two drug products: ▲*T* and *R*.▲ (USP 1-May-2019)The required studies and the determination of BE are the province of regulatory agencies. In the United States, *R* is termed the RLD and is so noted in FDA's *Approved Drug Products with Therapeutic Equivalence Evaluations,*▲37th Edition▲ (USP 1-May-2019) [*Orange Book* (▲2017▲ (USP 1-May-2019)) (http://www.accessdata.fda.gov/scripts/cder/ob/)]. To assist countries and regions where the *R* product may not always be readily identifiable, WHO has prepared a document titled *Annex 11: Guidance on the Selection of Comparator Pharmaceutical Products for Equivalence Assessment of Interchangeable Multisource (Generic) Products* (2002) (search by document title; http://www.who.int/en/). In the WHO document, *R* is termed the "comparator pharmaceutical product" (CPP). When a country or region has a clearly defined set of CPPs, the task becomes one of requiring that a manufacturer demonstrate, to the satisfaction of its regulatory authority, that its ▲▲ (USP 1-May-2019) product is pharmaceutically equivalent (PE) and ▲bioequivalent▲ (USP 1-May-2019) to the corresponding CPP.

Change to read:

BIOEQUIVALENCE

An interchangeable ▲drug▲ (USP 1-May-2019) product must be PE. The ▲EMA and▲ (USP 1-May-2019) WHO documents allow pharmaceutical alternatives to be considered ▲TE▲ (USP 1-May-2019) and interchangeable if they are ▲bioequivalent.▲ (USP 1-May-2019) Further, generic products must be shown to be bioequivalent to be considered ▲TE▲ (USP 1-May-2019) to the *R* product▲▲ (USP 1-May-2019). For the product to be considered PE, it must have the same ▲drug substance,▲ (USP 1-May-2019) same strength, same dosage form, same route of administration, and same labeling as the ▲*R*▲ (USP 1-May-2019) product. Several methods exist to assess and document BE. These include the following:

1. *Comparative pharmacokinetic studies in humans.* In these studies, the ▲drug substance▲ (USP 1-May-2019) and/or its metabolite(s) are measured as a function of time in accessible biological fluid such as blood, plasma, serum, or urine to obtain pharmacokinetic measures such as area under the plasma drug concentration versus time curve (AUC) and maximum concentration (C_{max}) that are reflective of systemic exposure. ▲Other pharmacokinetic parameters such as time to peak concentration (T_{max}) may be informative for safety and efficacy assessment.▲ (USP 1-May-2019)
BE studies are designed to compare the in vivo performance of ▲1)▲ (USP 1-May-2019) a generic product with an *R* product ▲or 2) a new formulation of an *R* product with the originally approved product.▲ (USP 1-May-2019) Generally the design is a two-period, two-sequence, single-dose, crossover randomized ▲study.▲ (USP 1-May-2019) The number of subjects should be statistically justified and NLT 12. ▲The necessary statistical power of the study will determine the number of subjects >12.▲ (USP 1-May-2019) During the study, blood samples are collected at sufficient intervals for assessing C_{max}, AUC, and other parameters. Blood samples are analyzed using appropriately validated bioanalytical methodology with standard pharmacokinetic measures and statistical approaches. The statistical method for testing pharmacokinetic BE is based on the determination of the 90% confidence interval around the geometric mean ratio of the log-transformed population means (▲*T*▲ (USP 1-May-2019)/*R*) for AUC and C_{max} by carrying out two one-sided tests at the 5% level of significance.
2. *Other options.* In addition, comparative pharmacodynamic studies in humans and comparative clinical trials can be used to document or supplement BE assessment. ▲▲ (USP 1-May-2019) In vivo documentation of equivalence is especially important for the following: drugs with a narrow therapeutic range; documented evidence of BE problems; modified-release pharmaceutical products designed to act by systemic absorption; and fixed-dose combination products with systemic action when at least one of the ▲drug substances▲ (USP 1-May-2019) requires an in vivo study.

Immediate-Release Drug Products

Single-dose, crossover BE studies are carried out at the highest dose comparing *T* and *R* products under fasting conditions. A parallel study design can be used for drugs that have a very long elimination half-life ($t_{½}$). Sampling truncation at 72 h may be allowable by regulatory agencies. Lower strength(s) of the dosage form can be given a biowaiver based on ▲linear pharmacokinetics,▲ (USP 1-May-2019) dosage form proportionality, and dissolution profile similarity. Food-effect studies are required ▲as described within scale-up and postapproval changes-immediate release (SUPAC-IR). Food effect studies are required for all *T* immediate-release oral products except where: 1) the *T* and *R* products are both rapidly dissolving with similar dissolution profiles and containing highly soluble and highly permeable drug substances; or 2) where the labeling of the *R* product states that the product must be taken on an empty stomach; or 3) where the *R* product labeling does not make a statement about

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food effect on absorption or administration [see FDA *Guidance for Industry: Food-Effect Bioavailability and Fed Bioequivalence Studies* (2002); (search by document title; http://www.fda.gov/)].▲ (USP 1-May-2019)

Modified-Release Drug Products

BE studies for ▲modified▲ (USP 1-May-2019)-release dosage forms are carried out as single-dose, crossover studies under fasting and fed conditions at the highest dose to compare *T* and *R* products. A single-dose study is more sensitive than multiple-dose, steady-state studies in assessing in vivo drug product performance, particularly with regard to the phenomenon of dose dumping, i.e., the rapid and unintended premature release of the ▲drug substance▲ (USP 1-May-2019) from an extended-release product into the ▲gastrointestinal tract.▲ (USP 1-May-2019) Lower strengths of an extended-release dosage form may not require an in vivo study based on use of the same drug-releasing mechanism, dosage form proportionality, and similar dissolution profile. ▲The FDA *Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation* (search by document title; http://www.fda.gov/) provides guidance on the need for BE studies when changes are made to the formulation. BE studies under fed conditions are required for all generic orally administered modified-release products [see FDA *Guidance for Industry: Food-Effect Bioavailability and Fed Bioequivalence Studies* (2002)].▲ (USP 1-May-2019)

Orally Administered Drug Products, Not for Systemic Effect

Some oral drug products are intended for local activity. Mesalamine and cholestyramine are examples of drugs that are intended for local activity. For these types of drugs, systemic absorption from the gastrointestinal tract is minimal; thus a comparative clinical trial is required while a systemic drug exposure profile also may be required. In some cases, in vitro studies may be appropriate, such as studies including comparison of cholestyramine binding to bile salts.

Bioequivalence Studies

Objective: The objective of a BE study is to measure and compare formulation performance between two or more ▲▲ (USP 1-May-2019) drug products. Drug availability from *T* and *R* products should not be statistically different when the drug is administered to patients or subjects at the same molar dose under similar experimental conditions.

Design: The design of a BE study depends on the objectives of the study, the ability to analyze the drug ▲substance (and/or▲ (USP 1-May-2019) metabolites ▲where appropriate)▲ (USP 1-May-2019) in biological fluids, the pharmacodynamics of the drug substance, the route of drug administration, and the nature of the drug and drug product. Pharmacokinetic parameters, pharmacodynamic parameters, clinical observations, and/or in vitro studies may be used to determine drug BA from a drug product.

Some possible BE study designs include the following:

1. Single-dose, two-way crossover study under fasted conditions
2. Single-dose, two-way crossover study under fed conditions
3. Single-dose, parallel study under fasted conditions
4. Single-dose, replicate design
5. Single-dose, partial replicate design
6. Multiple-dose, two-way crossover study, fasted conditions
7. Pharmacodynamic or clinical endpoint study
8. In vitro dissolution profile comparisons

The standard BE study is a crossover design (e.g., Latin square crossover design) in which each subject receives the ▲*T*▲ (USP 1-May-2019) drug product and the ▲*R* drug▲ (USP 1-May-2019) product on separate occasions. Studies are usually evaluated by a single-dose, two-period, two-treatment, two-sequence, open-label, randomized crossover design comparing equal doses of the ▲*T*▲ (USP 1-May-2019) and ▲*R*▲ (USP 1-May-2019) products in fasted or fed adult healthy subjects. A multiple-dose study may be required for some extended-release drug products. A washout ▲▲ (USP 1-May-2019) is scheduled between the two periods to allow the subjects to completely eliminate the drug absorbed from the first dose before administration of the second dose ▲of drug product.▲ (USP 1-May-2019) If the predose concentration is ≤5% of the C_{max} value in that subject, the subject's data without any adjustments can be included in all pharmacokinetic measurements and calculations. Samples of an accessible biologic fluid such as blood are used to characterize the drug concentration versus time profile. During the fasting study, subjects are fasted at least 10 h. A predose (0 time) blood sample is taken. The drug product is given with 240 mL (8 fluid ounces) of water. No food is allowed for at least 4 h post dose. ▲The *T* and *R* drug products are administered at the same time of day to avoid diurnal effects.▲ (USP 1-May-2019) Blood sampling is performed periodically after ▲▲ (USP 1-May-2019) administration ▲of drug product▲ (USP 1-May-2019) according to protocol. A food intervention or food effect study is conducted with standard meal conditions that are expected to provide the greatest effects on gastrointestinal physiology so that systemic drug availability is maximally affected. In addition, the high lipid content of the meal may affect the rate of drug release from the product, in situ. A high-fat (approximately 50% of total caloric content of the meal) and high-calorie (approximately 800–1000 calories) meal is recommended as a test meal for food-effect BA and fed BE studies. This test meal should derive approximately 150, 250, and 500–600 calories from protein, carbohydrate, and fat, respectively. The drug product is given with 240 mL (8 fluid ounces) of water after ingestion of the standard meal. Subjects should consume identical meals ▲within the same interval before administration of the *T* or *R* drug products.▲ (USP 1-May-2019)

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Analysis of samples: Samples, usually plasma, are analyzed for the ▲drug substance▲ (USP 1-May-2019) and, on occasion, active metabolite concentrations by a validated bioanalytical method.

Pharmacokinetic parameters: Pharmacokinetic parameters are obtained from the resulting concentration–time curves. Two major pharmacokinetic parameters are used to assess the rate and extent of systemic drug absorption. AUC reflects the extent of drug absorption, and the ▲C_{max}▲ (USP 1-May-2019) reflects the rate of drug absorption. Other pharmacokinetic parameters may include the ▲T_{max}▲ (USP 1-May-2019), the elimination rate constant (k), elimination half-life ($t_{½}$), lag time (T_{lag}), and others.

Statistical Analysis

Pharmacokinetic parameters are analyzed statistically to determine whether the T and R products yield comparable values. Because BE studies may use small sample sizes, log transformation of the data allows the frequency distribution of the data to be more normalized so that parametric statistical analyses may be performed (see FDA's *Guidance for Industry: Statistical Approaches to Establishing Bioequivalence* (2001); search by document title; http://www.fda.gov/).

Parametric (normal-theory) general linear model procedures are recommended for the analysis of pharmacokinetic data derived from in vivo BE studies. An analysis of variance (ANOVA) should be performed on the pharmacokinetic parameters AUC and C_{max} using appropriate statistical programs and models. For example, for a conventional two-treatment, two-period, two-sequence (2 × 2) randomized crossover study design, the statistical model often includes factors accounting for the following sources of variation:

- Sequence (sometimes called Group or Order)
- Subjects, nested in sequences
- Period (or Phase)
- Treatment (sometimes called Drug or Formulation)

The sequence effect should be tested using the [subject (sequence)] mean square from the ANOVA as an error term. All other main effects should be tested against the residual error (error mean square) from the ANOVA. The least-squares means (LSMEANS) statement should be used to calculate LSMEANS for treatments. Estimates should be obtained for the adjusted differences between treatment means and the standard error associated with these differences.

The statistical assumptions underlying the ANOVA are as follows:

- Randomization of samples
- Homogeneity of variances
- Additivity (linearity) of the statistical model
- Independence and normality of residuals

In BE studies, these assumptions can be interpreted as follows:

- The subjects chosen for the study should be randomly assigned to the sequences of the study.
- The variances associated with the two treatments, as well as between the sequence groups, should be equal or at least comparable.
- The main effects of the statistical model, such as subject, sequence, period, and treatment effect for a standard 2 × 2 crossover study, should be additive. There should be no interactions between these effects.
- The residuals of the model should be independently and normally distributed.

If these assumptions are not met, additional steps should be taken prior to the ANOVA, including data transformation to improve the fit of the assumptions or use of a nonparametric statistical test in place of ANOVA. However, the normality and constant variance assumptions in the ANOVA model are known to be relatively robust (i.e., a small or moderate departure from one or both of these assumptions will not have a significant effect on the final result). The rationale for log transformation is provided in FDA's *Guidance for Industry: Statistical Approaches to Establishing Bioequivalence* (2001) [search by document title; see FDA http://www.fda.gov/]. ▲▲ (USP 1-May-2019)

The two one-sided tests procedure: A testing procedure termed the two one-sided tests procedure is used to determine the comparability of geometric mean values for pharmacokinetic parameters measured after administration of the ▲T▲ (USP 1-May-2019) and ▲R▲ (USP 1-May-2019) products.[1] The two one-sided tests procedure determines whether T is not importantly less than R and whether R is not importantly less than T. ▲Other statistical approaches to the determination of a clinically meaningful difference may be used.▲ (USP 1-May-2019) Most often, 20% defines ▲a clinically meaningful▲ (USP 1-May-2019) difference. The statistical procedure involves the calculation of a confidence interval for the ratio (or difference) between T and R pharmacokinetic variable averages. The limits of the observed confidence interval must fall within a predetermined range for the ratio (or difference) of the product averages. Point estimate mean ratios (T/R) derived from the log-transformed AUC and C_{max} data must be between 80% and 125%. Because data are log transformed, T/R = 80/100 = 80% and R/T = 100/80 = 125%. In addition, the 90% confidence intervals for the geometric mean ratios (T/R) for AUC and C_{max} must be between ▲80.00%▲ (USP 1-May-2019) and ▲125.00%.▲ (USP 1-May-2019) The regulatory requirements for the range of 90% confidence intervals for C_{max} ▲are▲ (USP 1-May-2019) different in countries outside of the United States.

Bio-inequivalence: The failure to demonstrate BE may be due to a performance failure of the T product or to an inadequate study design. The failure to demonstrate BE because of an inadequate study design can be due to improper sampling in which: 1) the sampling time for C_{max} was not properly obtained, or 2) the number of samples taken did not adequately describe the plasma drug concentration versus time profile. Often, with highly variable drugs (e.g., % coefficient of variation [CV] ▲of C_{max} or AUC > 30%), where failure to demonstrate BE was observed, the study was not powered adequately due to too few subjects used.▲ (USP 1-May-2019)

[1] Schuirmann DJ. A comparison of the two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. *J Pharmacokinet Biopharm.* 1987;15(6):657–680.

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Presentation of data: The drug concentration in biological fluid at each sampling time point should be furnished untransformed for all the subjects who participated in the study. The derived pharmacokinetic parameters also should be furnished untransformed. The mean, the standard deviation, and the CV for each variable should be computed and tabulated in the final report.

To facilitate BE comparisons, pharmacokinetic parameters for each individual should be displayed in parallel for the formulations tested. In particular, for AUC and C_{max}, the difference ($T - R$), the ratio (T/R), and the log of ratio (log T/R or ln T/R) between the T and R values should be tabulated side-by-side for all the subjects. For each subject, the summary tables should indicate in which sequence (T then R, or R then T) the subject received the product. Histograms showing the frequency distribution of the difference and ln ratio (or log ratio) for the major pharmacokinetic parameters (AUC and C_{max}) are useful in the submission. ▲NMT 20% of the total AUC (zero to infinity) should be obtained by an extrapolated terminal elimination.▲ (USP 1-May-2019)

In addition to the arithmetic mean for the T and R products, the geometric means (antilog of the means of the logs), means of the logs, and standard deviations of the logs should be calculated for AUC and C_{max}. All means, including arithmetic mean, geometric mean, and means of the logs, as well as standard deviations and CVs, should be included in the report.

Change to read:

DISSOLUTION AND IN VITRO PRODUCT PERFORMANCE

As noted for an official preparation, *USP* monographs provide a public specification that includes a list of tests, references to analytical procedures, and acceptance criteria. Most solid oral dosage forms, including oral suspensions, require a dissolution or drug release test. Drug dissolution and drug release testing are described in ⟨711⟩ and ⟨724⟩. These public specifications are used for quality control tests and for market approval. The *USP* dissolution test in the monograph is related to BA and BE only when closely allied with a sound regulatory determination. Without this association, the *USP* dissolution test should be regarded solely as a quality control test for batch release. FDA guidances are: 1) *Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms*▲(1997)▲ (USP 1-May-2019) (http://www.fda.gov/; search by document title); ▲▲ (USP 1-May-2019) 2) *Guidance for Industry: Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations*▲(1997); and 3) *Guidance for Industry: Dissolution Testing and Specification Criteria for Immediate-Release Solid Oral Dosage Forms Containing Biopharmaceutics Classification System Class 1 and 3 Drugs* (draft July 2015)▲ (USP 1-May-2019) (search by document title; http://www.fda.gov/).

Dissolution and In Vitro BA

Drug dissolution and release tests are very useful during drug product development for identifying critical manufacturing attributes such as the impact of ingredient properties and the impact of the manufacturing process on drug product performance. During product development, optimum dissolution conditions need to be developed to discriminate among drug product formulations and changes in manufacturing processes. After the finished dosage form is approved for marketing, drug dissolution and release tests ▲may be▲ (USP 1-May-2019) useful in predicting possible changes in performance due to ▲scale-up and postapproval changes.▲ (USP 1-May-2019) See the following FDA guidances:

Guidance for Industry: Immediate Release Solid Oral Dosage Forms, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation (1995) (search by document title; http://www.fda.gov/) and

Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation (▲1997)▲ (USP 1-May-2019) (search by document title; http://www.fda.gov/).

For some oral drug products, in vitro drug dissolution can be related to in vivo performance, such as ▲BA▲ (USP 1-May-2019) and/or systemic drug exposure. Chapter ⟨1088⟩ describes various approaches to IVIVC.

Dissolution and In Vitro Equivalence

▲An appropriate▲ (USP 1-May-2019) dissolution test is a powerful in vitro physiochemical test that measures drug product quality and performance for a variety of dosage forms, such as solid oral dosage forms, transdermal dosage forms, suspensions, and certain semisolid dosage forms. The *USP* tests for finished dosage forms can be divided into two types: 1) drug product quality tests, and 2) drug product performance tests. Product quality tests are intended to assess attributes such as assay and content uniformity; product performance tests are designed to assess product performance, and in many cases relate to dissolution. For details regarding the performance of a dissolution▲/drug release▲ (USP 1-May-2019) test, see ⟨711⟩, ⟨724⟩, ⟨1088⟩, ▲▲ (USP 1-May-2019) ⟨1092⟩,▲and *Semisolid Drug Products—Performance Tests* ⟨1724⟩.▲ (USP 1-May-2019)

The in vitro dissolution test was initially developed as a quality control tool to ensure drug product quality and batch-to-batch consistency. The test procedures for conducting dissolution▲/drug release▲ (USP 1-May-2019) tests are described in ⟨711⟩ and ⟨724⟩. The development of the BCS brings new understanding and power to the dissolution test. The BCS classifies the drug substance according to ▲its aqueous▲ (USP 1-May-2019) solubility, ▲as well as its ▲ (USP 1-May-2019) permeability ▲▲ (USP 1-May-2019) through a biomembrane, such as the intestinal mucosal cells. The dissolution rate of the drug ▲substance▲ (USP 1-May-2019) from the dosage form is important in substantiating biowaivers based on the BCS.

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Dissolution Profile Comparisons

In vitro drug dissolution and release testing can be related to in vivo drug performance, such as BA. The comparisons of dissolution profiles are gaining importance as a means of documenting comparative BA studies—that is, BE. A biowaiver is the replacement or waiver ▲(USP 1-May-2019) of in vivo BE studies by an in vitro test.

A model independent mathematical approach is used to compare the dissolution profiles of two products: 1) to compare the dissolution profile between the *T*▲(USP 1-May-2019) product and *R*▲(USP 1-May-2019) product in biowaiver considerations; 2) to compare the dissolution profile between the two strengths of products from a given manufacturer; and 3) for SUPAC after the product is approved. For comparing the dissolution profile, the similarity factor f_2 should be computed using the equation:

$$f_2 = 50 \bullet \log\left\{\left[1+(1/n)\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} \bullet 100\right\}$$

where R_t and T_t are the cumulative percentage of the drug dissolved at each of the selected n time points of the ▲*R*▲(USP 1-May-2019) and ▲*T*▲(USP 1-May-2019) product, respectively. An f_2 value of 50 or greater (50–100) ensures dissolution profile similarity ▲▲(USP 1-May-2019) and thus ▲equivalence of▲(USP 1-May-2019) the performance of the two products. ▲FDA guidance recommends that, at▲(USP 1-May-2019) a minimum three points, NMT 1 point exceeding 85% ▲of the label claim dissolved,▲(USP 1-May-2019) should be used for similarity profile comparison. For products that dissolve very rapidly (≥85% dissolution in 15 min), a profile comparison is not necessary. ▲Different guidances have slightly different considerations in the application of dissolution profile comparisons that are given in *Table A-1*.▲(USP 1-May-2019)

Add the following:

▲INTERCHANGEABILITY OF DRUG PRODUCTS

The interchangeability of drug products is a major concern for physicians, pharmacists, and others who prescribe, dispense, or purchase them. Interchangeability of drug products is a regulatory decision requiring a determination of therapeutic equivalence. The FDA determines therapeutic equivalence of products in the U.S. Because the formulation and method of manufacture of the drug product can affect its BA and stability, the manufacturer must demonstrate that the *T* drug product is bioequivalent and *TE* to the *R*. The factors that can affect the drug product performance and therefore interchangeability of the finished drug product include:

- Solid form and particle size of the drug substance
- Differences in excipients in the formulation
- Differences in the manufacturing process▲(USP 1-May-2019)

Add the following:

▲SOLID FORM AND PARTICLE SIZE

As a result of the synthetic route and method of purification, the drug substance may be present with different particle size or solid form. Different solid forms, crystalline or amorphous, can dissolve at different rates, and therefore the extent of absorption and BA of these substances from the same dosage form, e.g., oral tablet, may be different. For example, crystalline structures are more thermodynamically stable than amorphous forms and as a consequence may dissolve more slowly. Different polymorph, hydrate, or salts of the drug substance may also have different physical chemical properties. These differing forms of the drug may have different aqueous solubilities or may dissolve at different rates. Particle size or particle size distribution of the drug may also affect the rate of dissolution. A drug substance having a particle size distribution with a large number of very fine particles may dissolve faster and lead to systemic absorption that is more rapid than for a particle size distribution with larger particles.▲(USP 1-May-2019)

Add the following:

▲DIFFERENCES IN EXCIPIENTS

A pharmaceutical dosage form typically consists of excipients as well as the drug substance(s). An excipient is any component, other than the drug substance(s), intentionally added to the formulation of a dosage form. Excipients, which may have no pharmacodynamic activity, play a critical role in manufacturing, stability, and performance. Excipients are manufactured to comply with compendial standards that address quality. However, the physical and chemical properties of the excipients affect the performance of a finished dosage form at least as much as the physical and chemical properties of the drug substance.▲(USP 1-May-2019)

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Add the following:

▲MANUFACTURING PROCESS

Variation in the manufacturing process can have a dramatic effect on drug product performance. Blending, sieving, compression force, precompression or granulation, and coating are all manufacturing operations that can impact product performance. Interchangeability can be affected by the manufacturing operation that is as important a consideration as the control of the drug substance form and the properties of the excipients used in the formulation.▲ (USP 1-May-2019)

Change to read:

BIOWAIVER

The term "biowaiver" is applied to a regulatory approval process when the application (dossier) is approved on the basis of evidence of equivalence other than an in vivo BE test. For solid oral dosage forms, the evidence of equivalence is determined on the basis of an in vitro dissolution profile comparison between the ▲*T*▲ (USP 1-May-2019) and the ▲*R*▲ (USP 1-May-2019) product. ▲▲ (USP 1-May-2019)

Biowaiver Based on Dosage Form Proportionality

When a single-dose fasting BE study is conducted on the designated (usually highest) strength of the drug product, the requirement for the conduct of additional in vivo BE studies on the lower strengths of the same product can be waived, provided that the lower strength: 1) is in the same dosage form; 2) is proportionally similar in its active and inactive ingredients; 3) has the same drug release mechanism (for extended-release products); 4) meets an appropriate in vitro dissolution profile comparison criterion (▲e.g.,▲ (USP 1-May-2019) $f_2 \geq 50$); and 5) both lower and higher strengths are within the linear pharmacokinetic range.

Biowaiver Based on the BCS

▲BCS provides a regulatory platform for replacing certain BE studies with in vitro dissolution tests.▲ (USP 1-May-2019) The BCS ▲▲ (USP 1-May-2019) takes into account three major factors that govern the rate and extent of drug absorption from immediate-release dosage forms. ▲These are aqueous solubility and intestinal permeability of the drug substance in conjunction with the dissolution of the pharmaceutical dosage form. These properties are assessed and categorized as being low or high for the purpose of classification.▲ (USP 1-May-2019) On the basis of the solubility and permeability of the dosage form, the drug substance is ▲categorized in different▲ (USP 1-May-2019) classes:

- Class 1: high solubility, high permeability
- Class 2: low solubility, high permeability
- Class 3: high solubility, low permeability
- Class 4: low solubility, low permeability

▲Solubility classification of a drug substance as low or high depends on the dose of the drug product. FDA defines a drug substance as being highly soluble "when the highest dose strength is soluble in 250 mL or less of aqueous media over the pH range of 1.0–6.8" (See *FDA Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* (December 2017) (search by document title; http://www.fda.gov/). However, EMA uses the highest single dose administered, and WHO uses the highest therapeutic dose approved by a competent authority, typically defined by the labeling for the innovator product.

The classification of drug substance permeability as high or low differs among the FDA, WHO, and EMA. FDA states that a drug substance is highly permeable where the extent of absorption is 90% or more of an administered dose in the absence of evidence of instability in the gastrointestinal tract. WHO considers a drug substance (API) highly permeable when the extent of absorption is 85% or more of an administered dose. EMA finds complete absorption to be established where the measured extent of absorption is ≥85% and goes on to say that complete absorption is generally related to high permeability. FDA, WHO, and EMA all use mass balance or comparison to an intravenous reference dose (EMA: absolute BA) in support of the claim of extent of absorption.

The FDA requirements for BCS-based biowaiver apply to immediate-release drug products when the drug substance is BCS Class 1 and the *R* and *T* products are rapidly dissolving (NLT 85% in NMT 30 min) or BCS Class 3 where *R* and *T* are very rapidly dissolving (NLT 85% in NMT 15 min) and contain the same excipients. *T* and *R* products should be PE and exhibit similar dissolution profiles. The FDA biowaiver excludes immediate-release products that have a narrow therapeutic index or are designed to be absorbed in the oral cavity. The EMA requirements for BCS-based biowaiver are applied to immediate-release solid oral drug products considered not to have a narrow therapeutic index. Both BCS Class 1 and 3 compounds can be considered. The *T* and *R* must have the identical drug substance, but different salts are acceptable as long as both belong within BCS Class 1. For BCS Class 1 drug substances, in vitro dissolution is either very rapid (i.e., >85% in 15 min) or similarly rapid (i.e., 85% in <30 min and similar dissolution profiles). For BCS Class 3 drug substances, a biowaiver would require very rapid in vitro dissolution for both *T* and *R*. Excipients that might affect BA are the same and other excipients are similar qualitatively and in similar amounts.

The WHO requirements for BCS-based biowaiver apply to BCS Class 1 and 3 drug substances. For BCS Class 1 substances, both *T* and *R* products should be rapidly dissolving and demonstrate similar dissolution profiles. For BCS Class

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3 drug substances, dissolution should be very rapid in vitro. The same excipient composition for both *T* and *R* drug products is required. ▲(USP 1-May-2019)

Use of the BCS has become a means of documenting BE without the conduct of an in vivo study; see the FDA guidance *Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* ▲(December 2017)▲ (USP 1-May-2019) (search by document title; http://www.fda.gov/).

The in vitro dissolution studies are generally carried out by basket method at 100 rpm or by paddle method at 50 rpm [FDA guidance cited immediately above ▲and EMA guidance, *Guideline on the Investigation of Bioequivalence* (2010)]▲ (USP 1-May-2019) or 75 rpm ▲[WHO guidance, *Annex 7: Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability*, WHO Expert Committee on Specifications for Pharmaceutical Products: Fortieth Report. WHO: Geneva; 2006: Annex 7 WHO Technical Report Series, No. 937). TRS 992]▲ (USP 1-May-2019) (search by document title; http://www.who.int/en/) in 900 mL of medium at pH 1.2, 4.5, and 6.8. On the basis of dissolution rate, the pharmaceutical dosage forms are classified as: 1) very rapidly ▲(FDA, EMA and WHO)▲ (USP 1-May-2019) dissolving, if 85% or more of the ▲labeled content▲ (USP 1-May-2019) dissolves in 15 min or less, or 2) rapidly dissolving, if 85% or more of the ▲labeled content▲ (USP 1-May-2019) dissolves in 30 min. ▲▲ (USP 1-May-2019)

For biowaiver, the dissolution tests should be carried out for both ▲*T*▲ (USP 1-May-2019) and ▲*R*▲ (USP 1-May-2019) product under the same test conditions. For the generic product to be eligible for biowaiver, the ▲*R*▲ (USP 1-May-2019) product should belong to the same BCS class and should meet dissolution profile comparison criteria. On the basis of the BCS classification and dissolution profile comparison, a biowaiver can be considered by regulatory authorities, provided the dissolution profile similarity criteria ▲▲ (USP 1-May-2019) are met ▲*Table A-1*).▲ (USP 1-May-2019)

DISSOLUTION AS A QUALITY CONTROL TEST AND ▲VERSUS AS AN IN VITRO EQUIVALENCE TEST▲ (USP 1-May-2019)

There is a clear difference between dissolution as a quality control test and dissolution as an in vitro equivalence ▲▲ (USP 1-May-2019) test. For immediate-release dosage forms, the quality control test involves a single-point dissolution test in only one medium (generally a compendial test). On the other hand, the in vitro equivalence test ▲▲ (USP 1-May-2019) involves dissolution profile comparison in pH 1.2, 4.5, and 6.8 between the *T* product and the *R* product.

Change to read:

APPENDIX

Comparisons Among FDA, EMA, and WHO

▲Table A-1. Comparison of Approaches to BCS-Based Biowaiver

	FDA	EMA	WHO
Conditions for f_2 calculation	Only one measurement should be considered after 85% dissolution of both the products	A minimum of 3 time points (0 excluded) and NMT 1 mean value of >85% dissolved for any of the formulations	A maximum of 1 time point should be considered after 85% dissolution of the reference (comparator product) has been reached. In cases where 85% dissolution cannot be reached, the dissolution should be conducted until an asymptote (plateau) has been reached.
Requirements	$f_2 \geq 50$	$50 \leq f_2 \leq 100$	$50 \leq f_2 \leq 100$
Very rapidly dissolving	NLT 85% in 15 min (using the method according to the guideline)	>85% of the labeled amount dissolves in 15 min (using the method according to the guideline)	NLT 85% of the labeled amount dissolves in 15 min (using the method according to the guideline)
Rapidly dissolving	≥85% of the labeled amount dissolves in 30 min (using the method according to the guideline)	≥85% of the labeled amount dissolves within 30 min (similarly rapid) (using the method according to the guideline)	NLT 85% of the labeled amount dissolves in 30 min (using the method according to the guideline)
Biowaiver	BCS Class 1 drug product (*T* and *R*) is rapidly dissolving, and *T* does not contain any excipients that would affect rate and extent of absorption of the drug. BCS Class 3: drug product (*T* and *R*) is very rapidly dissolving, and *T* is qualitatively very similar in composition to *R*.	BCS Class 1 drugs and either very rapid dissolution or similarly rapid. Excipients that might affect BA are qualitatively and quantitatively the same; BCS Class 3 drugs and very rapid dissolution and excipients that might affect BA are qualitatively and quantitatively the same; and other excipients are qualitatively the same and quantitatively very similar.	BCS Class 1: *T* and *R* are very rapidly dissolving or similarly rapidly dissolving drugs; BCS Class 3: *T* and *R* are very rapidly dissolving.
Apparatus	USP Apparatus 1 USP Apparatus 2	Basket Paddle	Basket Paddle

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Table A-1. Comparison of Approaches to BCS-Based Biowaiver *(continued)*

	FDA	EMA	WHO
Dissolution media	0.1 N hydrochloride or simulated gastric fluid (without enzymes) Buffer pH 4.5 Buffer pH 6.8 or simulated intestinal fluid (without enzymes)	pH 1.2 (0.1 N hydrochloride or SGF) Buffer pH 4.5 Buffer pH 6.8 or SIF	Buffer pH 1.2 Buffer pH 4.5 (acetate buffer) Buffer pH 6.8
	For capsules and tablets with gelatin coating, simulated gastric and intestinal fluids USP (with enzymes) can be used.	Absolutely no addition of surfactants. Use of enzymes is allowed for gelatin capsules.	International pharmacopoeia buffers are preferred
Volume	500 mL or less	900 mL or less	900 mL or less
Temperature	37 ± 0.5°	37 ± 1°	37°
Agitation	Apparatus 1: 100 rpm Apparatus 2: 50 rpm (or 75 rpm when appropriately justified)	Basket: 100 rpm Paddle: 50 rpm	Basket: 100 rpm Paddle: 75 rpm
Sample number	12	12	12
Sampling time	Sufficient number of intervals, e.g., 10, 15, 20, 30 min	10, 15, 20, 30, 45 min	10, 15, 20, 30, 45, 60 min▲ (USP 1-May-2019)

▲Table A-2.▲ (USP 1-May-2019) Comparison of FDA, ▲EMA,▲ (USP 1-May-2019) and WHO Definitions

Term	FDA	▲EMA▲ (USP 1-May-2019)	WHO
Pharmaceutical equivalents	Drug products are considered pharmaceutical equivalents if they contain the same active ingredient(s), are of the same dosage form, have the same route of administration, and are identical in strength or concentration. Pharmaceutically equivalent drug products are formulated to contain the same amount of active ingredient in the same dosage form and to meet the same or compendial or other applicable standards (strength, quality, purity, and identity); but they may differ in characteristics such as shape, scoring configuration, release mechanisms, packaging, excipients, expiration time, and, within certain limits, labeling.	▲Medicinal products are pharmaceutically equivalent if they contain the same amount of the same active substance(s) in the same dosage forms that meet the same or comparable standards. Pharmaceutical equivalence does not necessarily imply bioequivalence as differences in the excipients and/or the manufacturing process can lead to faster or slower dissolution and/or absorption.▲ (USP 1-May-2019)	Products are pharmaceutical equivalents if they contain the same molar amount of the same ▲API(s)▲ (USP 1-May-2019) in the same dosage form▲,▲ (USP 1-May-2019) if they meet comparable standards▲,▲ (USP 1-May-2019) and if they are intended to be administered by the same route. Pharmaceutical equivalence does not necessarily imply TE ▲as▲ (USP 1-May-2019) differences in the ▲API solid state properties, the▲ (USP 1-May-2019) excipients and/or the manufacturing process and ▲▲ (USP 1-May-2019) other variables can lead to differences in product performance.
Pharmaceutical alternatives	Drug products are considered pharmaceutical alternatives if they contain the same therapeutic moiety but are different salts, esters, or complexes of that moiety or are different dosage forms or strengths.	▲Medicinal products that contain different salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of the active moiety, or which differ in dosage form or strength▲ (USP 1-May-2019)	Products are pharmaceutical alternative(s) if they contain the same ▲▲ (USP 1-May-2019) active pharmaceutical moiety or moieties but differ in dosage form (e.g., tablets vs. capsules)▲, strength,▲ (USP 1-May-2019) and/or chemical form (e.g., different salts, different esters). Pharmaceutical alternatives deliver the same active moiety by the same route of administration but are otherwise not pharmaceutically equivalent. They may or may not be bioequivalent or TE to the comparator product.

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▲Table A-2.▲ (USP 1-May-2019) Comparison of FDA, ▲EMA,▲ (USP 1-May-2019) and WHO Definitions *(continued)*

Term	FDA	▲EMA▲ (USP 1-May-2019)	WHO
Therapeutic equivalents	Drug products are considered to be therapeutic equivalents only if they are pharmaceutical equivalents and if they can be expected to have the same clinical effect and safety profile when administered to patients under the conditions specified in the labeling.	▲A medicinal product is TE to another product if it contains the same active substance or therapeutic moiety and, clinically, shows the same efficacy and safety as that product, whose efficacy and safety have been established. In practice, demonstration of BE is generally the most appropriate method of substantiating therapeutic equivalence between medicinal products, which are pharmaceutically equivalent or pharmaceutical alternatives, provided they contain excipients generally recognized as not having an influence on safety and efficacy and comply with labeling requirements with respect to excipients. However, in some cases where similar extent of absorption but different rates of absorption are observed, the products can still be judged TE if those differences are not of therapeutic relevance. A clinical study to prove that differences in absorption rate are not therapeutically relevant may be necessary.▲ (USP 1-May-2019)	Two pharmaceutical products are considered to be TE if they are pharmaceutically equivalent or pharmaceutical alternatives and after administration in the same molar dose, their effects, with respect to both efficacy and safety, are essentially the same when administered to patients by the same route under the conditions specified in the labeling. ▲This can be demonstrated by appropriate equivalence studies, such as pharmacokinetic, pharmacodynamic, clinical, or in vitro studies.▲ (USP 1-May-2019)
BA (bioavailability)	This term means the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action.	▲The rate and extent to which a substance or its active moiety is delivered from a pharmaceutical form and becomes available in the general circulation (taking into consideration that the substance in the general circulation is in exchange with the substance at the site of action).▲ (USP 1-May-2019)	The rate and extent to which the ▲▲ (USP 1-May-2019) active moiety is absorbed from a pharmaceutical dosage form and becomes available ▲at the site(s) of action. Reliable measurements of active pharmaceutical ingredient concentrations at the site of action are not usually possible. The substance in systemic circulation, however, is considered to be in equilibrium with the substance at the site(s) of action. Based on pharmacokinetic and clinical considerations, it is generally accepted that in the same subject an essentially similar plasma concentration time-course will result in an essentially similar concentration time-course at the site(s) of action.▲ (USP 1-May-2019)
Bioequivalent drug products	This term describes pharmaceutical equivalent or pharmaceutical alternative products that display comparable BA when studied under similar experimental conditions.	▲Two medicinal products are bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and if their bioavailabilities after administration in the same molar dose are similar to such degree that their effects, with respect to both efficacy and safety, will be essentially the same.▲ (USP 1-May-2019)	Two pharmaceutical products are bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and their BA, in terms of ▲rate C_{max} and t_{max} and the extent of absorption (area under the curve [AUC])▲ (USP 1-May-2019) after administration of the same molar dose under the same conditions, are similar to such a degree that their effects can be expected to be essentially the same.
▲*R* drug product▲ (USP 1-May-2019)	An RLD [21 CFR 314.94(a)(3)] means the listed drug identified by FDA as the drug product upon which an applicant relies in seeking approval of its ANDA.	▲A reference medicinal product is a medicinal product authorized under Article 6, in accordance with the provisions of Article 8 (EC Directive 2001/83/EC)▲ (USP 1-May-2019)	The comparator ▲pharmaceutical product (CPP)▲ (USP 1-May-2019) is a pharmaceutical product with which the multisource product is intended to be interchangeable in clinical practice. The comparator product normally will be the innovator product for which efficacy, safety, and quality have been established. The selection of the comparator product usually is made at the national level by the drug regulatory authority.

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▲Table A-2.▲ (USP 1-May-2019) Comparison of FDA, ▲EMA,▲ (USP 1-May-2019) and WHO Definitions *(continued)*

Term	FDA	▲EMA▲ (USP 1-May-2019)	WHO
▲*T* drug product▲ (USP 1-May-2019)	A generic product is a product that is therapeutically equivalent to the RLD and is intended to be interchangeable with the innovator product.	▲A generic medicinal product which has the same qualitative and quantitative composition in active substances and the same pharmaceutical form as the reference medicinal product, and whose BE with the reference medicinal product has been demonstrated by appropriate BA studies. The different salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of an active substance shall be considered to be the same active substance, unless they differ significantly in properties with regard to safety and/or efficacy. In such cases, additional information providing proof of the safety and/or efficacy of the various salts, esters, or derivatives of an authorized active substance must be supplied by the applicant. The various immediate-release oral pharmaceutical forms shall be considered to be one and the same pharmaceutical form.▲ (USP 1-May-2019)	▲See *Multisource pharmaceutical products.*▲ (USP 1-May-2019)
Multisource pharmaceutical products			Pharmaceutically equivalent or pharmaceutically alternative products that may or may not be therapeutically equivalent. Multisource pharmaceutical products that are therapeutically equivalent are interchangeable.
Interchangeable pharmaceutical product			An interchangeable pharmaceutical product is one ▲which▲ (USP 1-May-2019) is therapeutically equivalent to a comparator product and can be interchanged with the comparator in clinical practice.

⟨1091⟩ LABELING OF INACTIVE INGREDIENTS

This informational chapter provides guidelines for labeling of inactive ingredients present in dosage forms.

Within the past few years a number of trade associations representing pharmaceutical manufacturers have adopted voluntary guidelines for the disclosure and labeling of inactive ingredients. This is helpful to individuals who are sensitive to particular substances and who wish to identify the presence or confirm the absence of such substances in drug products. Because of the actions of these associations, the labeling of therapeutically inactive ingredients currently is deemed to constitute good pharmaceutical practice.

Although the manufacturers represented by these associations produce most of the products sold in this country, not all manufacturers, repackagers, or labelers here or abroad are members of these associations. Further, there are some differences in association guidelines. The guidelines presented here are designed to help promote consistency in labeling.

In accordance with good pharmaceutical practice, all dosage forms [NOTE—for requirements on parenteral and topical preparations, see the *General Notices, 5.20.20 Added Substances (Excipients and Ingredients) in Official Products*] should be labeled to state the identity of all added substances (therapeutically inactive ingredients) present therein, including colors, except that flavors and fragrances may be listed by the general term "flavor" or "fragrance." Such listing should be in alphabetical order by name and be distinguished from the identification statement of the active ingredient(s).

The name of an inactive ingredient should be taken from the current edition of one of the following reference works (in the following order of precedence): (1) the *United States Pharmacopeia* or the *National Formulary;* (2) *USAN and the USP Dictionary of Drug Names;* (3) CTFA *Cosmetic Ingredient Dictionary;* (4) *Food Chemicals Codex*. An ingredient not listed in any of the aforementioned reference works should be identified by its common or usual name (the name generally recognized by consumers or health-care professionals) or, if no common or usual name is available, by its chemical or other technical name.

An ingredient that may be, but not always is, present in a product should be qualified by words such as "or" or "may also contain."

The name of an ingredient whose identity is a trade secret may be omitted from the list if the list states "and other ingredients." For the purposes of this guideline, an ingredient is considered to be a trade secret only if its presence confers a significant competitive advantage upon its manufacturer and if its identity cannot be ascertained by the use of modern analytical technology.

An incidental trace ingredient having no functional or technical effect on the product need not be listed unless it has been demonstrated to cause sensitivity reactions or allergic responses.

Inactive ingredients should be listed on the label of a container of a product intended for sale without prescription, except that in the case of a container that is too small, such information may be contained in other labeling on or within the package.

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⟨1092⟩ THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION

INTRODUCTION

Purpose

The Dissolution Procedure: Development and Validation ⟨1092⟩ provides a comprehensive approach covering items to consider for developing and validating dissolution procedures and the accompanying analytical procedures. It addresses the use of automation throughout the test and provides guidance and criteria for validation. It also addresses the treatment of the data generated and the interpretation of acceptance criteria for immediate- and modified-release solid oral dosage forms.

Scope

Chapter ⟨1092⟩ addresses the development and validation of dissolution procedures, with a focus on solid oral dosage forms. Many of the concepts presented, however, may be applicable to other dosage forms and routes of administration. General recommendations are given with the understanding that modifications of the apparatus and procedures as given in *USP* general chapters need to be justified.

The organization of ⟨1092⟩ follows the sequence of actions often performed in the development and validation of a dissolution test. The sections appear in the following sequence.

1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/DISSOLUTION METHOD DEVELOPMENT)
    - 1.1 Performing Filter Compatibility
    - 1.2 Determining Solubility and Stability of Drug Substance in Various Media
    - 1.3 Choosing a Medium and Volume
    - 1.4 Choosing an Apparatus
2. METHOD DEVELOPMENT
    - 2.1 Deaeration
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    - 2.4 Study Design
        - 2.4.1 Time Points
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4. AUTOMATION
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5.5 Robustness
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1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/ DISSOLUTION METHOD DEVELOPMENT)

Before method development can begin, it is important to characterize the molecule so that the filter, medium, volume of medium, and apparatus can be chosen properly in order to evaluate the performance of the dosage form.

1.1 Performing Filter Compatibility

Filtration is a key sample-preparation step in achieving accurate test results. The purpose of filtration is to remove undissolved drug and excipients from the withdrawn solution. If not removed from the sample solution, particles of the drug will continue to dissolve and can bias the results. Therefore, filtering the dissolution samples is usually necessary and should be done immediately if the filter is not positioned on the cannula.

Filtration also removes insoluble excipients that may otherwise interfere with the analytical finish. Selection of the proper filter material is important and should be accomplished, and experimentally justified, early in the development of the dissolution procedure. Important characteristics to consider when choosing a filter material are type, filter size, and pore size. The filter that is selected based on evaluation during the early stages of dissolution procedure development may need to be reconsidered at a later time point. Requalification has to be considered after a change in composition of the drug product or changes in the quality of the ingredients (e.g. particle size of microcrystalline cellulose).

Examples of filters used in dissolution testing can be cannula filters, filter disks or frits, filter tips, or syringe filters. The filter material has to be compatible with the media and the drug. Common pore sizes range from 0.20 to 70 µm, however, filters of other pore sizes can be used as needed. If the drug substance particle size is very small (e.g., micronized or nanoparticles), it can be challenging to find a filter pore size that excludes these small particles.

Adsorption of the drug(s) by the filter may occur and needs to be evaluated. Filter materials will interact with dissolution media to affect the recovery of the individual solutes and must be considered on a case-by-case basis. Different filter materials exhibit different drug-binding properties. Percentage of drug loss from the filtrate due to binding may be dependent on the drug concentration. Therefore the adsorptive interference should be evaluated on sample solutions at different concentrations bracketing the expected concentration range. Where the drug adsorption is saturable, discarding an initial volume of filtrate may allow the collection of a subsequent solution that approaches the original solution concentration. Alternative filter materials that minimize adsorptive interference can usually be found. Prewetting of the filter with the medium may be necessary. In addition, it is important that leachables from the filter do not interfere with the analytical procedure. This can be evaluated by analyzing the filtered dissolution medium and comparing it with the unfiltered medium.

The filter size should be based on the volume to be withdrawn and the amount of particles to be separated. Use of the correct filter dimensions will improve throughput and recovery, and also reduce clogging. Use of a large filter for small-volume filtration can lead to loss of sample through hold-up volume, whereas filtration through small filter sizes needs higher pressures and longer times, and the filters can clog quickly.

Filters used for USP Apparatus 4 need special attention because they are integrated in the flow-through process. Undissolved particles may deposit on the filters, creating resistance to the flow.

In the case of automated systems, selection of the filter with regard to material and pore size can be done in a similar manner to manual filtration. Flow rate through the filter and clogging may be critical for filters used in automated systems. Experimental verification that a filter is appropriate may be accomplished by comparing the responses for filtered and unfiltered standard and sample solutions. This is done by first preparing a suitable standard solution and a sample solution. For example, prepare a typical dissolution sample in a beaker and stir vigorously with a magnetic stirrer to dissolve the drug load completely. For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions.

1.2 Determining Solubility and Stability of Drug Substance in Various Media

Physical and chemical characteristics of the drug substance need to be determined as part of the process of selecting the proper dissolution medium. When deciding the composition of the medium for dissolution testing, it is important to evaluate the influence of buffers, pH, and if needed, different surfactants on the solubility and stability of the drug substance. Solubility

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of the drug substance is usually evaluated by determining the saturation concentration of the drug in different media at 37° using the shake-flask solubility method (equilibrium solubility). To level out potential ion effects between the drug and the buffers used in the media, mixtures of hydrochloric acid and sodium hydroxide are used to perform solubility investigations; this is in addition to the typical buffer solutions. In certain cases, it may be necessary to evaluate the solubility of the drug at temperatures other than 37° (i.e., 25°). The pH of the clear supernatant should be checked to determine whether the pH changes during the solubility test. Alternative approaches for solubility determination may also be used.

Typical media for dissolution may include the following (not listed in order of preference): diluted hydrochloric acid, buffers (phosphate or acetate) in the physiologic pH range of 1.2–7.5, simulated gastric or intestinal fluid (with or without enzymes), and water. For some drugs, incompatibility of the drug with certain buffers or salts may influence the choice of buffer. The molarity of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

Aqueous solutions (acidic or buffer solutions) may contain a percentage of a surfactant [e.g., sodium dodecyl sulfate (SDS), polysorbate, or lauryldimethylamine oxide] to enhance the solubility of the drug. The surfactants selected for the solubility investigations should cover all common surfactant types, i.e., anionic, nonionic, and cationic. When a suitable surfactant has been identified, different concentrations of that surfactant should be investigated to identify the lowest concentration needed to achieve sink conditions. Typically, the surfactant concentration is above its critical micellar concentration (CMC). *Table 1* shows a list of some of the surfactants used in dissolution media. Approximate CMC values are provided with references when available. The list is not comprehensive and is not intended to exclude surfactants that are not listed. Other substances, such as hydroxypropyl β-cyclodextrin, have been used as dissolution media additives to enhance dissolution of poorly soluble compounds. The U.S. Food and Drug Administration (FDA) maintains a database of dissolution methods, including information on dissolution media that have been used *(1)*. Typically, the amount of surfactant added is sufficient to achieve sink conditions in the desired volume of dissolution medium.

It is important to control the grade and purity of surfactants because use of different grades could affect the solubility of the drug. For example, SDS is available in both a technical grade and a high-purity grade. Obtaining polysorbate 80 from different sources can affect its suitability when performing high-performance liquid chromatography (HPLC) analysis.

There may be effects of counter-ions or pH on the solubility or solution stability of the surfactant solutions. For example, a precipitate forms when the potassium salt for the phosphate buffer is used at a concentration of 0.5 M in combination with SDS. This can be avoided by using the sodium phosphate salt when preparing media with SDS.

Table 1. Commonly Used Surfactants with Critical Micelle Concentrations

Surfactant		CMC (% wt/volume)	Reference
Anionic	Sodium dodecyl sulfate (SDS), Sodium lauryl sulfate (SLS)	0.18%–0.23%	*(2–4)*
	Taurocholic acid sodium salt	0.2%	*(3)*
	Cholic acid sodium salt	0.16%	*(3)*
	Desoxycholic acid sodium salt	0.12%	*(3)*
Cationic	Cetyltrimethyl ammonium bromide (CTAB, Hexadecyltrimethylammonium bromide)	0.033%–0.036% (0.92–1.0 mM)	*(5,6)*
	Benzethonium chloride (Hyamine 1622)	0.18% (4 mM)	*(2)*
Nonionic	Polysorbate 20 (Polyoxyethylene (20) sorbitan monolaurate, Tween 20)	0.07%–0.09%	*(3,7)*
	Polysorbate 80 (Polyoxyethylene (20) sorbitan monooleate, Tween 80)	0.02%–0.08%	*(3,7)*
	Caprylocaproyl polyoxyl-8 glycerides (Labrasol)	0.01%	*(4)*
	Polyoxyl 35 castor oil (Cremophor EL)	0.02%	*(8)*
	Polyoxyethylene 23 lauryl ether (Brij 35)	0.013%	*(9)*
	Octoxinol (Triton X-100)	0.01%–0.03%	*(3,10)*
Zwitterion	Lauryldimethylamine *N*-oxide (LDAO)	0.023%	*(11)*

Routinely, the dissolution medium is buffered; however, the use of purified water as the dissolution medium is suitable for products with a dissolution behavior independent of the pH of the medium. There are several reasons why purified water may not be preferred. The water quality can vary depending on its source, and the pH of the water is not as strictly controlled as the pH of buffer solutions. Additionally, the pH can vary from day to day and can also change during the run, depending on the drug substance and excipients. Use of an aqueous–organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

Investigations of the stability of the drug substance should be carried out, when needed, in the selected dissolution medium with excipients present, at 37°. This elevated temperature has the potential to decrease solution stability (degradation). Stability should allow for sufficient time to complete or repeat the analytical procedure. Physical stability may be of concern when precipitation occurs because of lower solubility at room or refrigerated temperature.

1.3 Choosing a Medium and Volume

When developing a dissolution procedure, one goal is to have sink conditions, which are defined as having a volume of medium at least three times the volume required to form a saturated solution of drug substance. When sink conditions are

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present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide sink conditions may be acceptable if it is appropriately justified. The composition and volume of dissolution medium are guided by the solubility investigations. For example, the choice and concentration of a surfactant need to be justified from the solubility data and the dissolution profiles.

The use of enzymes in the dissolution medium is permitted, in accordance with *Dissolution* ⟨711⟩, when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products. A discussion of the phenomenon of cross-linking and method development using enzymes can be found in *Capsules–Dissolution Testing and Related Quality Attributes* ⟨1094⟩. Validation should be performed with the method using enzymes according to section *5. Validation*.

Another option is to use media that follow more closely the composition of fluids in the stomach and intestinal tract. These media may contain physiological surface-active ingredients, such as taurocholates. The media also may contain emulsifiers (lecithin) and components such as saline solution that increase osmolality. Also, the ionic strength or molarity of the buffer solutions may be manipulated. The media are designed to represent the fed and fasted state in the stomach and small intestine. These media may be very useful in modeling in vivo dissolution behavior of immediate-release (IR) dosage forms, in particular those containing lipophilic drug substances, and may help in understanding the dissolution kinetics of the product related to the physiological make-up of the digestive fluids. Results of successful modeling of dissolution kinetics have been published, mainly for IR products. In the case of extended-release dosage forms with reduced effect of the drug substance on dissolution behavior, the use of such media needs to be evaluated differently. In vitro performance testing does not necessarily require media modeling the fasted and postprandial states *(12,13)*.

An acid stage is part of the testing of delayed-release products by *Method A* or *Method B* in ⟨711⟩. For drugs with acid solubility less than 10% of the label claim or drugs that degrade in acid the usefulness of the acid stage in detecting a coating failure is compromised. This would be handled on a case-by-case basis. Possible resolutions include the addition of surfactant to the acid stage, or adjustment of the specifications.

During selection of the dissolution medium, care should be taken to ensure that the drug substance is suitably stable throughout the analysis. In some cases, antioxidants such as ascorbic acid may be used in the dissolution medium to stabilize the drug. There are occasions where such actions are not sufficient. For compounds that rapidly degrade to form a stable degradant, monitoring the degradant alone or in combination with a drug substance may be more suitable than analyzing only the drug substance. In situ spectroscopic techniques tend to be less affected by degradation when compared with HPLC analysis (including UHPLC and other liquid chromatographic approaches).

For compendial Apparatus 1 (basket) and Apparatus 2 (paddle), the volume of the dissolution medium can vary from 500 to 1000 mL. Usually, the volume needed for the dissolution test can be determined in order to maintain sink conditions. In some cases, the volume can be increased to between 2 and 4 L, using larger vessels and depending on the concentration and sink conditions of the drug; justification for this approach is expected. In practice, the volume of the dissolution medium is usually maintained within the compendial range given above. Alternatively, it may be preferable to switch to other compendial apparatus, such as a reciprocating cylinder (Apparatus 3), reciprocating holder (Apparatus 7), or flow-through cell (Apparatus 4). Certain applications may require low volumes of dissolution media (e.g., 100–200 mL) when the use of a paddle or basket is preferred. In these cases, an alternative, noncompendial apparatus (e.g., small-volume apparatus) may be used.

1.4 Choosing an Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. In general, a compendial apparatus should be selected.

For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. When Apparatus 1 or Apparatus 2 is not appropriate, another official apparatus may be used. Apparatus 3 (reciprocating cylinder) has been found especially useful for chewable tablets, soft gelatin capsules, delayed-release dosage forms, and nondisintegrating-type products, such as coated beads. Apparatus 4 (flow-through cell) may offer advantages for modified-release dosage forms and immediate-release dosage forms that contain active ingredients with limited solubility. In addition, Apparatus 4 may have utility for multiple dosage form types such as soft gelatin capsules, beaded products, suppositories, or depot dosage forms, as well as suspension-type extended-release dosage forms. Apparatus 5 (paddle over disk) and Apparatus 6 (rotating cylinder) are useful for evaluating and testing transdermal dosage forms. Apparatus 7 (reciprocating holder) has application to non-disintegrating, oral modified-release dosage forms, stents, and implants, as well as transdermal dosage forms. For semisolid dosage forms, the generally used apparatus include the vertical diffusion cell, immersion cell, and flow-through cell apparatus with the insert for topical dosage forms (see *Semisolid Drug Products—Performance Tests* ⟨1724⟩).

Some changes can be made to the compendial apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10-, 20-, or 80-mesh) may be used when the need is clearly documented by supporting data. Care must be taken that baskets are uniform and meet the dimensional requirements specified in ⟨711⟩.

A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. A rotating bottle or dialysis tubes may have utility for microspheres and implants, peak vessels, and modified flow-through cells for special dosage forms including powders and stents.

2. METHOD DEVELOPMENT

A properly designed test should yield data that are not highly variable, and should be free of significant stability problems. High variability in the results can make it difficult to identify trends or effects of formulation changes. Sample size can affect the observed variability. One guidance defines dissolution results as highly variable if the relative standard deviation (RSD) is more than 20% at time points of 10 min or less and more than 10% at later time points for a sample size of 12 *(14)*. However, during method development, smaller sample sizes may be used, and the analyst will need to make a judgment accordingly. Most dissolution results, however, exhibit less variability. In the development of a dissolution procedure the source of the variability

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should be investigated, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing any of the following factors: the apparatus type, speed of agitation, level of deaeration, sinker type, or composition of the medium.

Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, excipient interactions or interference, film coating, capsule shell aging, and hardening or softening of the dosage form on stability may be sources of variability and interferences.

2.1 Deaeration

The significance of deaeration of the dissolution medium should be determined because air bubbles can act as a barrier to the dissolution process if present on the dosage unit or basket mesh and can adversely affect the reliability of the test results. Furthermore, bubbles can cause particles to cling to the apparatus and vessel walls. Bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. Poorly soluble drugs are most sensitive to interference from air bubbles; therefore, deaeration may be needed when testing these types of products. A deaeration method is described as a footnote in the *Procedure* section of ⟨711⟩. Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deaeration are available and are in routine use throughout the industry. Once a suitable deaeration process is identified, it should be documented as part of the dissolution procedure. The extent of deaeration can be evaluated by measuring the total dissolved gas pressure or by measuring the concentration of dissolved oxygen in water. For example, an oxygen concentration below 6 mg/L has been found effective as a marker for adequate deaeration of water for the Performance Verification Test with USP Prednisone Tablets RS.

Media containing surfactants usually are not deaerated because the process results in excessive foaming, and usually the effect of dissolved air on the dissolution process is mitigated by the reduced surface tension of the medium. Sometimes, deaerating the medium before adding surfactants can be effective.

To determine whether deaeration of the medium is necessary, compare results from dissolution samples run in non-deaerated medium and medium deaerated using a compendial technique, as described above. If no effect of deaeration is detected, this experiment could serve as justification that deaeration is not required in the future. If there is an effect, however, then it is necessary to carefully control this parameter, and it is prudent to characterize the robustness of the deaeration process. The dissolved gas content of deaerated media under atmospheric pressure is unstable and will tend toward saturation. Manipulations of the deaerated medium such as stirring or pouring can increase the rate at which atmospheric gases are redissolved.

2.2 Sinkers

Sinkers are often used to adjust the buoyancy of dosage forms that would otherwise float during testing with Apparatus 2. When sinkers are used, a detailed description of the sinker must be provided in the written procedure. It may be useful to evaluate different sinker types, recognizing that sinkers can significantly influence the dissolution profile of a dosage unit. When transferring the procedure, the same sinkers should be used, or if a different design is used, it should be shown to produce equivalent results. There are several types of commercially available sinkers. In ⟨711⟩, a harmonized sinker is described in *Figure 2a*.

A standard sinker can be made by using the appropriate length of wire and coiling it around a cylinder. For materials, use 316 stainless steel wire, typically 0.032 inch/20 gauge, or other inert material and wind the wire around cylinders of appropriate diameter (e.g., cork borers) for an appropriate number of turns to fit the capsule shell type. Sizes are shown in *Table 2*. The ends of the coil can be curved to retain the capsule within the sinker when they are immersed. Because the ends of the wire may be rough, they may need to be filed. If the sinker is handmade, the sinker material and construction procedure instructions should be documented (e.g., dimension, design, number of coils); if a commercial sinker is used, the vendor part number should be reported if available.

Table 2. Wire Sinkers Used With Common Capsule Shell Sizes

Capsule Shell Size	Length of Wire (cm)	Diameter Size (cm)	Cork Bore Number
#0, elongated	12	0.8	4
#1 and #2	10	0.7	3
#3 and #4	8	0.55	2

Although sinkers are typically used to keep the dosage form at the bottom of the vessel, they can also be used to keep dosage forms from sticking to the vessel (e.g., film-coated tablets). The sinker should be appropriate to the dosage form; therefore, the same sinker size may not be suitable for all dosage-form sizes. The sinker should not be too tight around the dosage form because this may restrict interaction with the medium. Conversely, if wrapped too loosely, the dosage form may escape soon after the test begins. The sinker should be small enough that the capsule does not change its orientation within the sinker. Care should be taken when testing capsules that have some cross-linking present, to keep the sticky shell from attaching to the vessel bottom. In this case, the harmonized sinker design provided in *Figure 2a* of ⟨711⟩ will be advantageous.

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2.3 Agitation

For immediate-release capsule or tablet formulations, Apparatus 1 (baskets) at 50–100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are used commonly. Other agitation speeds are acceptable with appropriate justification. Rates outside 25–150 rpm for both the paddle and the basket are usually not appropriate because of mixing inconsistencies that can be generated by stirring too slow or too fast. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions.

For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and improving the data. If justified, 100 rpm may be used with Apparatus 2, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if to achieve an in-vitro–in-vivo correlation (IVIVC) the resulting profiles better reflect in vivo performance, or if the method results in better discrimination without adversely affecting method variability.

Apparatus 3 (reciprocating cylinder) can be used at dip rates ranging from 5 to 30 dips/min. The hydrodynamics are influenced by the cylinder's reciprocating motion and the resulting movement of the sample in the medium. The reciprocating motion of the cylinder and screen may cause foaming if the medium contains surfactants. Addition of an anti-foaming agent such as simethicone or *n*-octanol may be useful for avoiding foaming from surfactants.

Apparatus 4 (flow-through cell) is described in ⟨711⟩ with standard flow rates of 4, 8, and 16 mL/min. Other flow rates for Apparatus 4 can be used if justified and if within the capacity of the pump to conform with the requirements in ⟨711⟩. Agitation in Apparatus 4 is not only related to the pump speed but can also be affected by cell diameter. At a set flow rate, as measured by volume, the 12-mm cell will develop a greater linear fluid velocity than is achieved in the 22.6-mm cell. Apparatus 4 can be configured with the addition of glass beads in the entry cone of the flow-through cell (packed column) or without glass beads (open column).

The flow characteristics of the flow-through cell are discussed in the scientific literature (*15*). The placement of the sample in the flow-through cell will influence the flow patterns that occur and thus should be a consideration in the attempt to reduce variability of the results.

2.4 Study Design

Selection of the agitation rate and other study design elements for the dosage form, whether immediate release or modified release, should conform to the requirements and specifications (i.e., apparatus, procedures, and interpretation) given in ⟨711⟩.

2.4.1 TIME POINTS

For immediate-release dosage forms, the duration of the dissolution procedure is typically 30–60 min; in most cases, a single time point specification is adequate for pharmacopeial purposes. For method development, however, a sufficient number of time points should be selected to adequately characterize the ascending and plateau phases of the dissolution curve. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. According to the Biopharmaceutics Classification System referred to in several FDA Guidances, highly soluble, highly permeable drugs formulated into very rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the drug substance within 15 min. For these types of products, a one-point test or disintegration will suffice. However, most products do not fall into this category. Dissolution profiles of immediate-release products typically show a gradual increase reaching 85%–100% at about 30–45 min. Thus, sufficient dissolution time points are chosen to characterize the performance for most immediate-release products. For some products, including suspensions, useful information may be obtained from earlier points, e.g., 5–10 min. For slower-dissolving products, time points later than 60 min may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

The f_2 similarity factor may not be useful when more than 85% is dissolved at 15 min. If the f_2 similarity factor is to be used, multiple time points for the dissolution test are required, with at least two time points with mean percent dissolved (typically for $n = 12$) below 85% dissolved and only one point above 85% for both products (*16*). Therefore, the addition of early time points may be useful.

For testing an extended-release dosage form, at least three time points are chosen, to guard against dose dumping, to define the in vitro release profile, and to show that essentially complete release (>80%) of the drug is achieved. Additional sampling times may be useful. Certain IVIVC criteria, such as level B correlation (according to *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩), require the experimental determination of the time to dissolve 100% of the label claim. Selection of the final time points is reflective of the data from the drug release profile that are generated during development. For products containing more than a single active ingredient, determine the drug release for each active ingredient.

Delayed-release dosage forms usually require specifications for at least two time points; therefore, it is important during development to evaluate the entire dissolution profile. In the case of enteric-coated dosage forms, the functionality of the coating is usually proven by challenge in an acid medium, followed by a demonstration of dissolution in a higher-pH medium. Chapter ⟨711⟩ gives a standard buffer medium for that stage of testing but other media may be used if justified. The timing of the acid stage is typically 2 h, and release in the buffer is similar to the timing for immediate-release forms. For delayed-release dosage forms that are not enteric coated, setting of specifications is different. Unlike delayed release, the onset of release is not determined by the experimental design, which is the pH change; multivariate specifications, therefore, may be needed to define time ranges and corresponding percentage ranges.

So-called infinity points can be useful during development studies. To obtain an infinity point, the paddle or basket speed is increased at the end of the run (after the last time point) for a sustained period (typically, 15–60 min), after which time an additional sample is taken. Although there is no requirement for 100% dissolution in the profile, the infinity point can be compared to content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

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2.4.2 OBSERVATIONS

Visual observations and recordings of product dissolution and disintegration behavior are useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. For visual observation, proper lighting (with appropriate consideration of photo-degradation) of the vessel contents and clear visibility in the bath are essential. Documenting observations by drawing sketches and taking photographs or videos can be instructive and helpful for those who are not able to observe the real-time dissolution test. Observations are especially useful during method development and formulation optimization. It is important to record observations of all six vessels to determine if the observation is seen in all six vessels, or just a few. If the test is performed to assist with formulation development, provide any unique observations to the formulator. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus (e.g., below the basket or paddle), when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit, especially at the surface of the media.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products, [e.g., the partial opening and splitting apart (similar to a clamshell) or incomplete opening of the shell], accompanied by the release of air bubbles and excipients.
9. Whether the dosage form lands in the vessel center or off-center, and if off-center, whether it sticks there.
10. Time required for the complete dissolution of the capsule shell or for tablet disintegration.

Observations also help to document that the proper procedure has been followed, or more importantly, that a deviation has occurred. Examples include the confirmation that a dosage form is actually in the vessel during the test or that more than one dosage form are inadvertently in the same vessel, or that a filter from the autosampler has dropped into the vessel.

2.4.3 SAMPLING

Manual: For manual sampling, use chemically inert devices (e.g., polymeric or glass syringes, and polymeric or stainless steel cannula), a filter, and/or a filter holder. The sampling site must conform to specifications in ⟨711⟩. When the agitation conditions are very slow, e.g., a 50-rpm basket, care should be taken to sample consistently in the same location in the vessel because there may be a concentration gradient; avoid sampling very close to the shaft or vessel wall. During method development, a decision should be made regarding whether to replace the media after each time point. Replacement is not preferred because the dosage unit may be disturbed during delivery of the media. However, replacement may be necessary if maintaining sink conditions is a challenge. With replacement, the volume used in the calculations remains the same throughout the time points, but there is some drug substance withdrawn with each sample that will need to be accounted for in the calculations.

Metal surfaces may interact with the sample. For example, adsorption onto metal surfaces may occur, or the metal surfaces may release metal ions into aqueous media. The ions can then catalyze degradation reactions, leading to artifacts during the analytical procedures. The surfaces of stirring elements and metal locks of syringes may be sources of interference to accurate sampling.

Autosampling: Autosampling is discussed in section *4. Automation*.

2.4.4 CLEANING

Importance is placed on evaluation of the cleaning process between tests. Changes of dissolution medium and/or product necessitate the need for cleaning. Residues on the vessels can affect the results (e.g., adsorbed residues may dissolve and alter subsequent media properties or interfere with the sample analysis), and effective cleaning will return them to a suitable state. Automated systems are discussed in section *4.4 Cleaning*.

Change to read:

2.5 Data Handling

Dissolution rates are calculated from the change in drug concentration in the dissolution medium. For procedures in which the volume of medium is fixed, such as for Apparatus 1 and Apparatus 2 testing of immediate-release dosage forms with only one sampling time, the concentration of the sample is multiplied by the medium volume to arrive at the mass of drug dissolved usually expressed as percentage of label claim. When multiple time points are taken, the total amount of drug removed at earlier time points should be assessed and may be part of the calculation of the amount dissolved, if considered important. Similarly, if the medium volume is not fixed, for example when the sample volume is not replaced in testing extended-release products, the change in medium volume must be part of the calculation for successive sampling points. Dissolution tests performed with Apparatus 4 in the closed-loop configuration with in situ detection provide a convenient control of the medium volume. For testing with Apparatus 4 in the open configuration, the test time and flow rate will determine the volume of medium used in the dissolution calculations.

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Dissolution results can be evaluated as either cumulative rates or fractional rates. Cumulative rates represent the sum of all drug dissolution that occurs during an interval (*Figure 1*). Fractional rates are assessed at a specific time point or during a portion of the total test time (*Figure 2*). Typically, the rate of release will be expressed as either mass or percentage of label claim per unit time. For most compendial dissolution testing, the dissolution rate is expressed as a percentage of the label claim dissolved at the indicated test time.

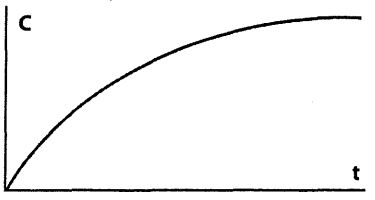

Figure 1. An example of a plot of dissolution as a cumulative process. Concentration, *C*, is the amount of drug released per volume of medium, and t represents time. This type of plot is readily observed in constant-volume dissolution systems, such as Apparatus 1 or Apparatus 2, or Apparatus 4 in closed-loop configuration.

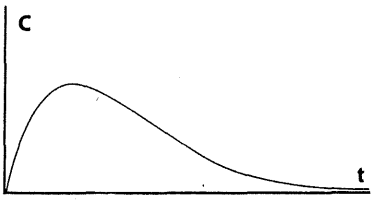

Figure 2. An example of a plot of the observed concentration of the sample taken for an interval that is negligibly small in relation to the time of the overall dissolution process. This concentration is propostional to the instantaneous or fractional dissolution rate (*dc*/*dt*). This type of plot is readily observed in continuous-flow dissolution systems, such as Apparatus 4 in open-loop configuration.

Cumulative dissolution profiles represent the total amount of drug dissolved from the formulation over time. When cumulative dissolution is measured in a constant-volume system, no correction for the amount lost in sampling needs to be made. If sample is removed from the system, the amount consumed in analysis must be accounted for in the calculation. Recirculated sampling with Apparatus 1 or Apparatus 2, or with Apparatus 4 in the closed-loop configuration (*Figure 3*), are all examples of systems that will produce cumulative dissolution rates. With Apparatus 4 in the open configuration (*Figure 4*), cumulative rates accounting for the total amount of drug dissolved across the testing interval are obtained by collecting and analyzing the entire outflow from each individual flow-through cell. With Apparatus 3 (*Figure 5*), the medium in each tube is sampled at the end of the programmed interval, and the analyzed concentration represents the cumulative dissolution rate during that interval.

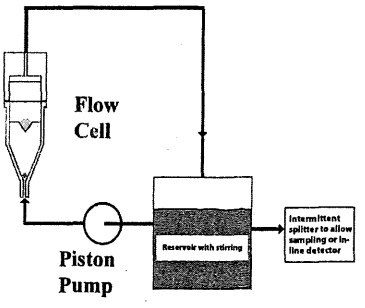

Figure 3. Apparatus 4 in the closed-loop configuration.

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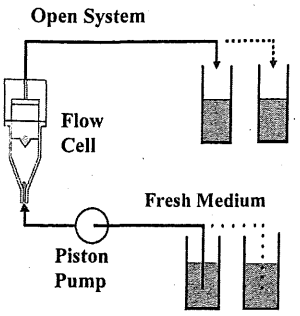

Figure 4. Apparatus 4 in the open-loop configuration. The sample can be collected in fractions, as shown at the top. The medium can be changed by using successive reservoirs.

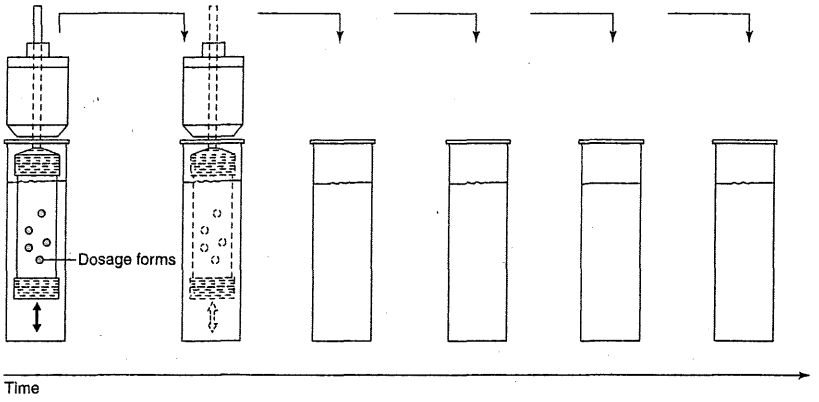

Figure 5. The progression that is possible for one reciprocating cylinder from Apparatus 3. The reciprocating cylinder can move from vessel to vessel. This feature facilitates changing the dissolution medium and testing for different intervals in successive tubes.

Fractional dissolution rates are typically measured for a discrete interval. A series of such rates will produce a step function as the dissolution profile. At any time, the cumulative dissolution rate from this type of profile is the sum of the preceding intervals. This type of profile is represented by Apparatus 3 using multiple tubes or Apparatus 4 in the open-loop configuration where the total outflow is collected and analyzed for successive intervals.

A number of algebraic and numerical methods exist for transforming cumulative and fractional dissolution results. The difference in amount released for successive time points can be calculated, and the average release rate is determined by the formula:

$$\text{Result} = (M_2 - M_1)/(t_2 - t_1)$$

M = mass or percentage of label claim
t = time

As the difference of t_2 from t_1 is reduced, the average rate can be considered to approach an instantaneous rate. Sampling considerations and physical constraints on measurement of the mass transfer at the medium interface of the dosage form make the measurement of true instantaneous dissolution impractical for routine determination in the laboratory. Fractional dissolution is measured for intervals where the difference between t_2 and t_1 is small, relative to the total test time. The design of Apparatus 4 in the open configuration permits a direct measurement of the fractional dissolution over small time intervals. For example, if a 4-mL fraction of outflow for Apparatus 4 running 16 mL/min is sampled, either by in situ detection or offline, the amount of drug detected represents the dissolution occurring in a 15-s interval.

Pooled dissolution has been used in a number of monographs. The pooled dissolution procedure produces an average release rate for the units tested by combining equal volumes from each vessel or cell and performing analysis of only the one resulting solution. Because this approach uses only the average release rate for comparison with the acceptance table, the pooled dissolution procedure has been viewed as reducing the amount of data available from the dissolution test and, thus, reducing its value. However, it should be noted that the pooling of equal sample volumes is equivalent, from a calculation standpoint, to determining the arithmetic mean of the individual sample results.

The use of the f_2 similarity factor in the comparison of dissolution profiles is discussed in ▲*Assessment of Solid Oral Drug Product Performance and Interchangeability, Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩▲ (CN 1-May-2019).

For the purpose of correlation with in vivo data, parameters of mathematical models are obtained by fitting to dissolution data to establish a continuous functional relationship called IVIVC (see ⟨1088⟩).

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2.6 Dissolution Procedure Assessment

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that together provide a method that is sensitive to changes in critical quality attributes, yet sufficiently rugged and reproducible for day-to-day operation. The method should be able to be transferred between laboratories.

The ideal dissolution procedure will not contribute an unacceptable degree of variability and will provide a profile with adequate points below 85% dissolved. If 85% dissolved occurs before 15 min, then f_2 comparisons may not be appropriate.

There are many ways to challenge the sensitivity of the method. One option is to compare dissolution profiles of formulations that are intentionally manufactured with meaningful variations for the most relevant critical manufacturing variable, for example, ±10%–20% change to the ranges of these variables. Similarly, samples that have been stressed may be used to demonstrate sensitivity to changes on stability. This concept may be used to establish the factors that are most significant in their influence on the dissolution rate. These studies can focus on either the dissolution parameters (e.g., media concentration, agitation rate, and deaeration) or the product attributes (e.g., excipient ratios, particle size, compression). The ultimate goal is to understand the release mechanisms and determine whether the dissolution procedure can show change in the critical quality attributes of a drug product.

3. ANALYTICAL FINISH

The dissolution step has been described as an involved sample preparation. The sample handling and analytical procedure that are used to determine the amount of drug substance dissolved during the dissolution procedure are termed the "analytical finish." Although spectrophotometric determinations and HPLC are used most commonly and are discussed in this chapter, any suitable analytical technology may be used. Section *5. Validation* describes criteria for the methods.

3.1 Sample Processing

After the samples are withdrawn from the dissolution medium, they may require additional processing to make them suitable for the analytical methodology used to determine the amount released. For example, filtration may be used to remove undissolved particulate matter, or samples may need to be protected from exposure to light or may need refrigerated storage. In addition, samples may have to be diluted to a level that is within the linear range of the method. With analysis by HPLC, dilution of the sample with mobile phase may be necessary to reduce the effect on the separation of injecting dissolution medium. Other types of treatment may be necessary depending on the product formulation, such as the inactivation or elimination of interference caused by components of the formulation by the addition of appropriate reagents. However, separation may not be possible or needed in all cases. In some cases, in situ measurements obtained with methods such as fiber optics or electrochemical determination may be useful.

3.2 Filters

The topic of filtration is discussed in section *1.1 Performing Filter Compatibility*.

3.3 Centrifugation

Centrifugation of samples is not preferred, for several reasons: dissolution can continue to occur until the solids are removed, a concentration gradient may form in the supernatant, and energy imparted may lead to increased dissolution of the drug substance particles. Possible exceptions, when centrifugation could be preferred, might include the use with compounds that adsorb onto all common filters, or situations when the potential filter leachables and extractables might interfere in the quantitative step of the dissolution test (e.g., when fluorescence procedures are used in quantitation). Centrifugation may prove useful during method development for evaluating the suitability of the filter material.

3.4 Analytical Procedure

The usual assay for a dissolution sample employs either a spectrophotometric procedure or a liquid chromatographic procedure. Spectrophotometric determination may be direct or may provide the detection for HPLC. Spectrophotometric determination is used often because results can be obtained faster, the analysis is simpler, it is easier to automate, and fewer solvents are needed. The use of direct spectrophotometric determination typically requires confirmation of specificity. HPLC is preferred for a number of reasons such as providing a wide dynamic range that reduces the need to dilute some samples while also providing sensitivity in the analysis of dilute samples, and greater selectivity when excipients or multiple drugs in the formulation present a significant interference. Modern HPLC systems employ autosamplers that provide speed and simplicity advantages comparable to spectrophotometric analysis.

3.5 Spectrophotometric Analysis

Direct spectrophotometric analysis may be performed on samples that are manually introduced to the cuvette. Alternatively, samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance, as described in the standard operating procedures or metrology documents, help to ensure reliable operation of these instruments. Cells with path lengths ranging from 0.02 cm to 1 cm are typically used, and longer path-length cuvettes can be used to increase the range for quantification of dilute samples. Cell alignment and air bubbles

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could be sources of error. The shorter path-length cells are used to avoid diluting the sample; in all cases, however, acceptable linearity and standard error need to be demonstrated.

The choice of wavelength for the determination should be based on the spectrum of the drug in solution. In some cases, where the drug substance can degrade in the dissolution medium (e.g., dosage forms containing aspirin), it is useful to carry out the measurements at the isosbestic point. Excipients can also have effects, but performing analysis at multiple wavelengths can minimize their effects. The contribution of the absorbance from an excipient at the analytical wavelength can sometimes be determined by ratio from its absorbance at a wavelength where the absorbance of the drug substance is minimal.

Using a validated analytical finish, standard solutions are typically prepared in dissolution media and analyzed at just one concentration, either at 100% of the dosage strength or the selected *Q* value because linearity of the analytical finish has been established. Prior to validation, dissolution profile analysis, or analysis of products of various strengths, requires using multiple standard solutions covering the expected range of concentration. A typical media blank, standard, and sample may be analyzed in a sequence that brackets the sample with standards and blanks, especially at the beginning and end of the analysis. The standard and sample solutions should both be prepared in the dissolution medium in the linear concentration range and measured at the same wavelength. However, small amounts of an organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg/mL, divided by the cell path length in cm. A rearrangement of the Beer-Lambert expression gives the absorptivity, *a*, as:

$$a = A/bc$$

A = absorbance
b = path length (cm)
c = concentration (mg/mL)

Typical units for absorptivity that are used for dissolution testing are in terms of AU · mL/mg, where AU is absorbance unit. Historical data may be used to provide an acceptable absorptivity range for the analyte (using the appropriate path-length cell). This value may be useful in troubleshooting aberrant data.

Fiber optics as a sampling and determinative method, with proper validation, are an option.

3.6 HPLC

For HPLC analysis, the effect on the chromatogram of peaks resulting from injection of dissolution media require enumeration. A large solvent disturbance may affect accuracy and precision of response if it is poorly resolved from the peak of interest. This is even more important if large injector volumes (>100 µL) are needed. System suitability tests may evaluate peak shape; separation of the main peak from solvent disturbance and from closely eluting peaks; and injection precision. At a minimum, the precision is critical.

Ideally, the standard solutions should be diluted with the dissolution media at a concentration within the linear range of the method, e.g., 100%, or the selected *Q* value of the dosage strength. However, organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation. In some cases, the sample may be diluted with mobile phase to improve the peak shape. The standard and sample solutions should both be prepared in the linear concentration range and measured at the same wavelength.

4. AUTOMATION

Automated dissolution systems may be configured in various ways and degrees. The elements of test preparation, initiation, sampling and timing, and cleaning all can be automated. Fully automated systems are available, as are systems where individual steps, such as media preparation or sampling, are automated. This section will discuss operational steps that can be automated. The level of complexity for automation depends on whether the instrument configuration is open or closed loop and also whether the analytical device is coupled online or offline. Online analysis returns the sample aliquot to the test system, as in the case of spectrophotometry with flow-through cuvettes. Offline analysis removes the sample aliquot from the dissolution medium for subsequent analysis, typically by HPLC, where the analysis consumes the sample. The decision on the configuration usually depends on the number of samples to be processed and the time required for their analysis.

Automation may require deviations from the pharmacopeial specifications of the instruments, such as incorporation of an integrated outlet on the bottom of the vessel for cleaning and replacement of medium.

Operational steps that are not part of the compendial procedure should be validated. Deviations from the standard procedure described in ⟨711⟩, such as use of sampling probes or fiber-optic probes, should be validated against the standard procedure.

4.1 Medium Preparation

Automated media preparation generally is accomplished by diluting concentrates. Automated media preparation systems typically dispense the volume of medium into the vessel by monitoring either the weight or volume. Chemical and physical stability of the concentrates as well as homogeneity of the dilutions over the intended period of use are important issues and should be understood. Concentrates of buffer solutions and surfactants may have stability issues, such as chemical degradation and pH change. Physical instability may manifest as precipitation, re-crystallization, or phase separation and should be prevented.

If deaeration of the medium is required, the level of deaeration should be specified.

The concentration of the dissolved oxygen can be used to evaluate the efficiency of deaeration procedures discussed in section *2.1 Deaeration*.

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4.2 Sample Introduction and Timing

Samples should be inserted in the vessel in a reproducible way. Automated sample introduction and aliquot withdrawal provide an advantage over manual sampling because the automated techniques can reduce the variability in the vessel-to-vessel timing of the test intervals. However, automated sample handling may impose timing limitations that need to be considered. The pharmacopeial tolerance of ±2% of the specified dissolution test time may be difficult to meet for early time points.

4.3 Sampling and Filtration

Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. The transfer and filtration of sample solutions from the dissolution instrument to the analytical unit may be undertaken via tube connections or via robotic devices operated in a stepwise procedure. Sample volumes may be removed from the dissolution medium and not returned (consumptive sampling), or the sample volume may be returned to the dissolution medium (recirculated sampling).

There are many brands of autosamplers, including semi-automated and fully automated systems. Routine performance checks, cleaning, and maintenance, as described in the pertinent standard operating procedures or metrology documents, help to ensure reliable operation of these devices.

Sampling probes may or may not remain in the vessel throughout the entire run. Sampling probes or fiber-optic probes can disturb the hydrodynamics of the vessel; therefore, adequate validation should be performed to ensure that the probes are not causing a significant change in the dissolution rate. If filters are used that are different from those used for manual sampling, then these different filters should also be evaluated separately. The position of the pharmacopeial sampling zone for Apparatus 1 and Apparatus 2 is midway from the top of the stirring element to the medium surface and depends on the medium volume. Sampling probes should pull the sample from the sampling zone. Instruments for which the sampling occurs through the hollow shaft should be designed with a means to adjust the depth of the inlet aperture to allow conformance with this requirement. The programmed sampling volume depends on the dead volume of the tubing, cuvettes, and other devices and has to be adjusted accordingly.

A recirculated sampling alignment can be operated either by discharging the tubing contents into the vessel after each sampling or by allowing the tubing to remain filled with solution in the intervals between sampling points. In the latter case, the dead volume and carryover effects are important considerations.

The need for sample volume replacement should be considered. In consumptive sampling with multiple sampling time points, the withdrawn volume may be replaced with an equal volume of fresh medium. The sampling volume may be critical if, in total, it exceeds 1% of the stated volume of dissolution medium required by the procedure. If it can be shown that replacement of the medium is not necessary, the volume change must be part of the calculation of results. See section *2.5 Data Handling*.

Carryover may occur when subsequent samples are affected by residues or conditions of previous samples; the effect of the first sample or condition "carries over" to the second. In liquid handling, residues of liquids previously in the sample solution may contaminate subsequent sample solutions. Dissolution media containing surfactants or lipids may present problems. Carryover may occur for successive samples taken over a multiple time-point test, as well as at the beginning of a new test due to the cleaning solution. This topic is discussed in section *4.4 Cleaning*.

Interaction of dissolved drug substance with the sampling and transfer devices is an important consideration. When adsorption of the dissolved drug substance occurs, it most often involves surfaces of the dissolution apparatus or sampling filters and tubing. Adsorption may be pH dependent in the case of charged, dissolved drug substance. Adsorption of the dissolved drug to the parts of the sampling device should be assessed using a typical sample solution (dissolution sample from the product or drug substance with formulation matrix) with known concentration. The typical design is a cross-validation with aliquots of the same sample solution passing and bypassing the sampling device (including the sampling probe, filter, tubing, valves, and pump). There is no general recommendation that may give preference to any kind of material or equipment construction (e.g., glass or specific polymers). See section *5.7 Considerations for Automation* for more information.

In addition to the information in section *2.4.3 Sampling*, connections of pumps and tubing may be sources of contamination in automated systems. Interferences with the spectroscopic analytical procedures, which are commonly used for dissolution testing, are less of a concern. However, interferences must be evaluated if the product under investigation contains low-dose metal salts, as do some dietary supplements.

Liquid transfer usually is undertaken via polymeric tubing. Inert materials such as polytetrafluoroethylene (PTFE) sometimes cannot be used because of their mechanical properties. Where flexible tubes are required, for example in peristaltic pumps or for coiling in a small radius, polypropylene (PP) or high-density polyethylene (HDPE) may be the preferred materials. Depending on the type of polymer and its crystallinity and density, leaching of constituents, mainly plasticizers, may occur. Leachables can interfere with the analytical procedure. The concentration leached to the sample solution usually depends on the surface, the temperature, the exposure time, the hydrodynamic conditions, and the composition of the media.

4.4 Cleaning

In addition to the information in section *2.4.4 Cleaning*, automated systems have specific cleaning issues. For example, evaluation of the effectiveness of purging and rinsing between sampling times and within-run condition of the tubing is recommended. Also it is important to evaluate the cleaning process between tests.

4.5 Operating Software and Computation of Results

The software systems for data evaluation and instrument operation must be validated as per 21 CFR 11 *(17)*.

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4.6 Common Deviations from the Compendial Procedures That May Require Validation

Some common areas of deviation from compendial procedures include the following:

- Sample introduction relative to start of spindle rotation
- Residence time and positioning of sampling probes
- Recirculated versus consumptive sampling
- Sample volume replacement in consumptive sampling.

5. VALIDATION

The validation topics described in this section are typical but not all-inclusive and can be viewed in the context of *Validation of Compendial Procedures* ⟨1225⟩, as well as the International Conference on Harmonization (ICH) document, *Validation of Analytical Procedures (18)*. Validation for both parts of the dissolution procedure, the analytical finish and the dissolution step, will be discussed in this section. The dissolution step is the release of the drug in the dissolution medium and sampling. The analytical finish is defined in section *3. Analytical Finish*. Validation of the analytical finish will evaluate the attributes, linearity and range, precision, specificity, accuracy/recovery, robustness, and stability of the sample and standard solutions. Validation of the dissolution step will include evaluation of precision and robustness of the dissolution sample preparation. Validation of the analytical finish is performed either using a standard solution or spiked placebo or by the method of standard addition (spiked drug product as described in *Accuracy* in ⟨1225⟩), as specified in the sections below. Validation of the dissolution step requires the use of a well-characterized dosage form (e.g., having tight content uniformity and uniform performance). Depending on the parameter of interest, validation of the sample handling and analytical procedure can be performed in situ, e.g., within the dissolution vessel. The validation parameters addressed and the extent of the validation may vary, depending on the phase of development or the intended use for the data.

The acceptance criteria are presented as guidelines only, and may differ for some products. Manufacturers should document the appropriate acceptance criteria for their products in pertinent Standard Operating Procedures (SOPs) or in validation protocols. Other considerations may be important for special dosage forms. Validation studies should be performed across the range of profile time points. For products containing more than a single active ingredient, the dissolution procedure needs to be validated for each active ingredient. It is expected that investigations into filter suitability and the potential for glass adsorption will have been undertaken already (see *1.1 Performing Filter Compatibility*). Validation of these assessments may occur during spiked recovery experiments.

5.1 Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by placebo constituents, other active drugs, or degradants. The placebo consists of all the excipients and coatings, with inks and capsule shells included if appropriate, without the active ingredient. Placebo interference can be evaluated by using a spiked placebo that is prepared by weighing samples of the placebo blend, dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing, and adding a known amount of the drug in solution. It may be preferable to perform this experiment at 37°, comparing the solution to a standard solution at the concentration expected to be encountered during testing, by using the formula:

$$\text{Result} = (A_P/A_S) \times C_S \times (V/L) \times 100$$

A_P = absorbance of the placebo
A_S = absorbance of the standard
C_S = concentration of the standard (mg/mL)
V = volume of the medium (mL)
L = label claim (mg)

The interference should not exceed 2%. Note that for extended-release products, a placebo version of the finished dosage form may be more appropriate than blends because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile, with worst-case interference expected at the later sampling points.

The blank is the dissolution medium without dissolved sample, and it is treated in the same manner as the sample. The effect of the absorbance of the blank at the analytical wavelength should be evaluated. In most cases, the absorbance of the dissolution medium blank may not exceed 1% of the standard solution at the concentration used for analysis. Values >1% should be evaluated on a case-by-case basis.

If the placebo interference exceeds 2%, modification of the method may be necessary. Possible modifications include choosing another wavelength, subtracting baseline using a longer wavelength, transforming absorbance values (e.g., first derivative), and using an alternative analytical technique such as HPLC. Other means for minimizing the placebo interference would be acceptable with appropriate justification. When other active drug substances or significant levels of degradants are present, it is necessary to show that these do not significantly affect the results. One procedure for doing this is to measure the matrix in the presence and absence of the other active drug substance or degradant: any interference should not exceed 2%. Similar approaches may be used if other techniques are used for the analytical finish.

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5.2 Linearity and Range

Linearity is typically established by preparing solutions of the drug substance, ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. The solutions may be prepared either using either a standard solution or spiked solution or by the method of standard addition. A minimum of five concentrations is normally used (see ⟨1225⟩). Typically, solutions are made from a common stock if possible. The concentration range may not exceed the linearity limits of the method, including the instrumentation. Organic solvents may be used to enhance drug solubility for the preparation of the linearity standard solutions. However, no more than 5% (v/v) of organic solvent should be present in the final solution unless validated. Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient ($r^2 \geq 0.98$) demonstrates linearity. In addition, the *y*-intercept must not be importantly different from zero.

The range of the procedure is the interval between the upper and lower concentrations of the drug substance (including these levels) that has been demonstrated to have a suitable level of precision, accuracy, and linearity using the procedure as written.

5.3 Accuracy/Recovery

Accuracy/recovery is typically established by preparing multiple samples containing the drug substance and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. Accuracy/recovery may be done in conjunction with linearity determination. The method of standard addition can also be used. Before this activity, it is expected that filter assessment will already have been performed, and adsorption of drug onto the glass has also been investigated and ruled out.

Individual solutions may be directly prepared in the dissolution medium. Alternatively, to enhance drug solubility it may be appropriate to prepare a stock solution by dissolving the drug substance in a small amount of organic solvent (typically not exceeding 5% organic solvent in the final dissolution media) and diluting to the final concentration with dissolution medium. An amount of stock solution equivalent to the targeted label claim may be used instead of the drug substance powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts.

The measured recovery is typically 95%–105% of the amount added. Bracketing or matrixing of multiple strengths may be useful. A special case for validation is the *Acid Stage* procedure described in ⟨711⟩, *Delayed-Release Dosage Forms*. The limit of NMT 10% needs to be validated. Recovery experiments for drugs that have low solubility in acidic media may be challenging or impossible to perform and may need to be addressed on a case-by-case basis. If the compound degrades in acid, the validation experiment must address this fact.

5.4 Precision

5.4.1 REPEATABILITY OF ANALYSIS

For the analytical finish, repeatability is evaluated by obtaining replicate measurements of standard and/or spiked placebo/standard addition solutions. It can be determined by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or by using the accuracy or linearity data. ICH guidance, *Validation of Analytical Procedures: Methodology*, recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration. A typical acceptance criterion is an RSD of <2%. The demonstration of the repeatability for the dissolution step is conducted by performing the dissolution step on separate units of a well-characterized dosage form or equivalent composite.

5.4.2 INTERMEDIATE PRECISION/RUGGEDNESS

Assuming that the major contributor to the variance is from the dissolution step, intermediate precision may be evaluated to determine the effects of random events on the precision of the dissolution procedure. This evaluation is typically done later in the development of the drug product and is required for full method validation. For many analytical procedures intermediate precision is typically assessed by determination of contributions to variance and, possibly, by a comparison of means. The use of an experimental matrix design is encouraged for evaluation of intermediate precision because interaction effects may be observed more clearly relative to a single variable experiment. In dissolution testing, a ruggedness approach that compares means alone is often taken to investigate the factors that contribute to intermediate precision. The ruggedness can be evaluated across the range of product strengths. Typical variations to be studied include different days, analysts, and equipment. If possible, ruggedness can be evaluated using a drug product lot if well characterized, for example, by having tight content uniformity and uniform performance, but if this type of lot is not available, a premeasured placebo with active ingredients may be used to investigate the intermediate precision. The use of such a spiked placebo would additionally support the assessment of the contribution of the analytical finish to the observed variability of results.

The dissolution procedure on the same lot of well-characterized dosage form may be run by at least two different analysts from the same laboratory, with each analyst preparing the standard solutions and the medium and following the defined extraction/quantification procedure. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers, and they perform the test on different days. Full profiles are assessed where relevant to the product. This procedure may not be necessary at each strength; instead, bracketing with high and low strengths may be acceptable.

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Acceptance criteria for intermediate precision or for ruggedness are predetermined. A typical acceptance criterion for ruggedness is that the difference in the mean value for dissolution results between any two conditions, using the same strength, does not exceed an absolute 10% at time points with <85% dissolved and does not exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

5.4.3 REPRODUCIBILITY

Reproducibility follows the general concepts of intermediate precision, but is performed by two different analysts at different labs.

5.5 Robustness

Evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in development of the drug product and is a requirement for full method validation. It is performed using a well-characterized lot of drug product, for example having tight content uniformity and uniform performance. The number of replicates (typically 3 or 6) is dependent on the intermediate precision. All profile points should be evaluated.

Selection of parameters to be varied depends on the dissolution procedure and analysis type. The parameters may include medium composition (e.g., buffer or surfactant concentration, pH, deaeration), volume, agitation rate, sampling time, and temperature. Statistical analysis of the data generated will help determine the extent to which the parameters must be controlled in the method. The robustness assessment is well suited to Design of Experiments (DoE) methodologies to efficiently investigate the impact of the individual parameters and/or their interaction.

Robustness of analytical finish is referenced in ⟨1225⟩. HPLC analysis parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

5.6 Stability of Standard and Sample Solutions

The standard solution is stored under conditions that ensure stability. The stability of the standard solution is analyzed over a specified period of time (for at least the time of the entire dissolution procedure), using a freshly prepared standard solution at each time interval for comparison. The acceptable range for standard solution stability is influenced by the concentration and is typically between 98% and 102% at the expected final concentration.

The sample solution is typically stored at room temperature. The sample is analyzed over a specified period of time, using the original sample solution response for comparison. The typical acceptable range for sample solution stability may be between 98% and 102%, compared with the initial analysis of the sample solutions. If the solution is not stable, aspects to consider include temperature (refrigeration may be needed), light protection, and container material (plastic or glass).

The procedure may state that the standards and samples need to be analyzed within a time period demonstrating acceptable standard and sample solution stability.

5.7 Considerations for Automation

Automated methods offer opportunities for increased precision and reproducibility; however, bias may be introduced. In particular, the sampling probe and the sample lines warrant attention as places where inaccuracies may occur. Deviations from the procedure described in ⟨711⟩, such as resident sampling probes, sampling through the stirring element shaft (hollow-shaft sampling), or fiber-optic probes, should be validated. Other aspects of automation validation may include carryover of residual drug, effect of an in-residence probe, adsorption of drug, and cleaning and/or rinse cycles. Validation is performed using the automated dissolution system including materials. Therefore, any change in materials will require demonstration of suitability based on the validation attributes that are impacted by the change.

Manual and automated procedures should be compared to evaluate the interchangeability of the procedures. This is done by performing two automated runs at each dosage concentration, using all sampling points, compared to manually sampled runs of the same samples. The effect of the in-resident probe cannot be determined by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision if the procedures are to be considered interchangeable. The difference in the mean value for dissolution results between any two conditions using the same strength should not exceed an absolute 10% at time points with <85% dissolved nor exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

Revalidation may be necessary when the automated system is used with different formulations because of the interaction with excipients. Dissolution media containing surfactants or lipids may require additional validation efforts.

6. ACCEPTANCE CRITERIA

The acceptance criteria should be consistent with historical release or stability data. There is an expectation that acceptable batches will have results that fall within the acceptance criteria and that all manufactured batches should have similar dissolution behavior, thus highlighting the importance of having a method that is not highly variable. The acceptance criteria and time point(s), therefore, should discriminate between an acceptable and an unacceptable batch. In addition, the dissolution test results are viewed as a link to the pivotal clinical trial batches. When changes in dissolution rate have been shown to affect bioavailability significantly, the dissolution test and acceptance criterion should distinguish batches with unacceptable bioavailability *(19)*. Likewise, when changes in the formulation and manufacturing process significantly affect dissolution and

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such changes are not controlled by another aspect of the specification, the dissolution test and criteria should distinguish these changes.

6.1 Immediate-Release Dosage Forms

Although release and stability data are collected during dosage form development, it is common to record the entire dissolution profile or the amount of drug dissolved at specified intervals, such as 10, 20, 30, 40, 50, and 60 min or 15, 30, 45, and 60 min. At registration, dissolution for an immediate-release tablet usually becomes a single-point test. The acceptance criterion and test time are established by evaluating the dissolution profile data. The acceptance criterion for a dissolution test is a function of *Q*, which is expressed as a percentage of label claim of drug dissolved at a specified time. Typical *Q* values are in the range of 75%–80% dissolved. *Q* values in excess of 80% are not generally used because allowance needs to be made for assay and content uniformity ranges.

6.2 Delayed-Release Dosage Forms

The discussion about dissolution of delayed-release dosage forms in ⟨711⟩ focuses on enteric-coated dosage forms, which is the most common delayed-release dosage form. A dissolution test for a delayed-release tablet or capsule is a two-part test, and each part has acceptance criteria. First, the dosage forms are exposed to an acid medium, followed by exposure to a buffer medium. To ensure that the enteric coating performs properly, a "NMT" acceptance criterion is indicated in ⟨711⟩ for the acid stage. The medium used for an acid stage is usually 0.1 N HCl, and the duration of this stage is typically 2 h. The dosage forms are then exposed to a buffer medium, usually 0.05 M phosphate buffer at pH 6.8, but other buffers and pH targets may be used if justified. The duration of the buffer stage is usually 45 min for compendial tests, but this duration may vary, depending on the drug product. As with immediate-release dosage forms, a *Q* value and time point are determined by evaluating the entire dissolution profile.

6.3 Extended-Release Dosage Forms

A dissolution test for an extended-release dosage form is generally similar to that used for an immediate- or delayed-release drug product, except that the duration of the test is longer, and at least three time points are specified for pharmacopeial purposes *(20)*. Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug *(20)*. The time points for the test should be determined by evaluating the dissolution profile across the desired test duration. Often, additional time points are obtained during dosage form development to aid with selecting the appropriate time points for the specification or monograph.

As with an immediate- or delayed-release drug product, the acceptance criteria and time points for an extended-release drug product should discriminate between an acceptable and an unacceptable batch. The acceptance criteria for the first stage of testing (L_1) should be established on the basis of available batch data *(19,20)*. If human bioavailability data are available for formulations exhibiting different release rates, then an in vitro/in vivo relationship may be used to establish acceptance criteria *(19,20)*. Acceptance criteria for the second (L_2) and third (L_3) stages are derived from the L_1 criteria using *Acceptance Table 2* in ⟨711⟩.

6.4 Multiple Dissolution Tests

Typically, monographs for extended-release dosage forms contain multiple dissolution tests representing specific products. In accordance with *General Notices, 4.10.10 Applicability of Test Procedures*, the appropriate test, if not *Test 1*, is indicated on the product labeling. For example, the *USP* monograph for *Oxycodone Hydrochloride Extended-Release Tablets (21)* lists two dissolution tests, each of which has either three or four time points. If the Tablets are analyzed using *Test 2* and the dissolution results comply with the criteria provided in the monograph, the labeling for Tablets can indicate that the Tablets meet USP *Dissolution Test 2*. Multiple dissolution tests also can be found in monographs for immediate- and delayed-release dosage forms. For example, the *USP* monographs for *Levothyroxine Sodium Tablets* and *Pantoprazole Sodium Delayed-Release Tablets* provide four dissolution tests *(22,23)*.

6.5 Interpretation of Dissolution Results

The *Interpretation* section of ⟨711⟩ discusses immediate-, delayed-, and extended-release dosage forms. The discussion for each of these release patterns is expanded here with examples to assist with applying the criteria during the various stages of testing. Understanding how these criteria are applied will assist in setting appropriate acceptance criteria.

6.5.1 IMMEDIATE-RELEASE DOSAGE FORMS

Once the *Q* value is established, the dissolution test is a staged test of three levels. In the first level of testing called S_1, six dosage forms are tested. Each dosage form must be *Q* + 5% (absolute percentage points) dissolved at a specified time. For example, the time and tolerances in a monograph would be:

Time: 30 min

Tolerances: NLT 80% (*Q*) of the labeled amount of "drug substance" is dissolved.

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If the *Q* value for a 200-mg label claim (LC) immediate-release tablet is specified as 80% and the time point is 30 min, then NLT 85% LC (170 mg) of the drug substance in each tablet must be dissolved at 30 min.

If this criterion is not met, then 6 additional tablets are tested at level 2 (S_2). To pass the S_2 acceptance criteria, the average of all 12 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example), and no tablet has less than *Q* – 15% (65% LC; 130 mg in the above example).

If these criteria are not met, then level 3 or S_3 testing must be performed by testing 12 additional tablets. To pass S_3, the average of all 24 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example). Two additional criteria must be met as well: 1) no more than 2 tablets are less than *Q* – 15% (65% LC; 130 mg in the above example), and 2) no tablet is less than *Q* – 25% dissolved (55% LC; 110 mg in the above example.)

6.5.2 DELAYED-RELEASE DOSAGE FORMS

An aliquot of the acid medium from each vessel is analyzed at the end of the acid stage. For the acid stage, the acceptance criteria have three levels. Level 1 (A_1) testing is passed if no individual value exceeds 10% dissolved. If the A_1 criteria are not met, then the dissolution test is performed on 6 additional dosage forms for level 2 (A_2) testing. Level A_2 criteria are passed if the average of all 12 dosage forms in the acid stage is NMT 10% dissolved and if no individual dosage form is more than 25% dissolved. Level 3 testing is performed if the A_2 criteria are not met. The A_3 criteria are passed if the average of all 24 dosage forms in the acid stage is NMT 10% dissolved and if no individual tablet is more than 25% dissolved. For the special case in which the solubility of the drug in an acidic medium because of conversion to the free acid is too low to support an acceptance criterion of not more than 10% the drug product should be exposed to the acid stage for the defined duration and then exposed to the buffered medium. Alternate acceptance criteria for the acid stage based on drug solubility may be justified.

For delayed-release dosage forms, the total percentage dissolved is determined by adding the measured amounts in the acid and buffer phases for each individual dosage form. These calculated values are then compared to staged acceptance criteria (B_1, B_2, and B_3) that are based on a *Q* value. The B_1, B_2, and B_3 criteria are identical to those for the immediate release S_1, S_2, and S_3 criteria.

6.5.3 EXTENDED-RELEASE DOSAGE FORMS

In the following hypothetical example, which is used to describe the criteria for an extended-release dosage form, the time points are 1, 4, and 8 h. The acceptance range for each time point is as follows:

- Between 24% and 44% LC drug substance dissolved at 1 h
- Between 56% and 76% LC drug substance dissolved at 4 h
- NLT 85% LC drug substance dissolved at 8 h.

Acceptance ranges are often expressed in tabular form in the *USP–NF* (see *Table 3*):

Table 3. L_1 Criteria

Time (h)	Amount Dissolved
1	24%–44%
4	56%–76%
8	NLT 85%

Six tablets are analyzed at Level 1 (L_1); acceptance criteria are met if no individual value lies outside each of the stated ranges, and no individual value is less than the percentage specified for the final time point. If the L_1 criteria are not met, then 6 additional tablets are analyzed at level 2 (L_2). The L_2 criteria are met if these three conditions are met:

1. The average value of the 12 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. None of the 12 tablets is >10% of the labeled content outside each of the stated ranges.
3. None of the 12 tablets is >10% of the labeled content below the stated amount at the final test time.

For the above example, the L_2 acceptance criteria for the 12 tablets (see *Table 4*) are as follows:

Table 4. L_2 Criteria

	1 h	4 h	8 h
Average	24%–44%	56%–76%	NLT 85%
Individual tablets	14%–54%	46%–86%	NLT 75%

If the L_2 criteria are not met, then 12 additional tablets are tested at level 3 (L_3). The L_3 criteria are met if these five conditions are met:

1. The average value of the 24 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. NMT 2 of the 24 tablets are >10% of labeled content outside each of the stated ranges.
3. NMT 2 of the 24 tablets are >10% of the labeled content below the stated amount at the final test time.
4. None of the 24 tablets is >20% of the labeled content outside each of the stated ranges.

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5. None of the 24 tablets is >20% of the labeled content below the stated amount at the final test time.

The L_3 acceptance criteria for the 24 tablets in the above example are summarized in *Table 5*:

Table 5. L_3 Criteria

	1 h	4 h	8 h
Average	24%–44%	56%–76%	NLT 85%
Individual Tablets	NMT 2 tablets are outside the range of 14%–54%, and no individual tablet is outside the range of 4%–64%	NMT 2 tablets are outside the range of 46%–86%, and no individual tablet is outside the range of 36%–96%	NMT 2 tablets release <75% and no individual tablet releases <65%

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⟨1094⟩ CAPSULES—DISSOLUTION TESTING AND RELATED QUALITY ATTRIBUTES

1. INTRODUCTION

This general information chapter provides approaches for the development of dissolution test procedures for capsules, which are not provided by *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *The Dissolution Procedure: Development and Validation* ⟨1092⟩, and *Disintegration and Dissolution of Dietary Supplements* ⟨2040⟩. The chapter also discusses quality attributes associated with capsules that may affect the outcome of the dissolution testing.

1.1 Types of Capsules

Capsules can be classified as two main types based on the physical characteristics of the shell: soft capsules and hard capsules. For the purpose of this chapter, soft-shell capsules and hard-shell capsules are referred to as softgels and hardgels, respectively. Softgels have a thicker shell and typically exhibit a higher degree of elasticity because of the added plasticizer and have slightly longer rupture time when compared with hardgels. By comparison, hardgel capsules have a thinner and more rigid shell than do softgel capsules. Both softgels and hardgels are composed of a polymer, e.g., gelatin, starch, or a cellulose derivative such as hypromellose (HPMC) or other polymers, a plasticizer, and water. For hardgels, water acts as the plasticizer, whereas softgels contain high-boiling-point polyols such as glycerol or sorbitol as plasticizer, and also contain water. Although many parameters affect the physical and chemical properties of the shell, the ratio of polymer to plasticizer primarily determines the rigidity, brittleness, and dissolution performance of the shell.

Capsules can also be characterized by the chemical properties of the fill material (hydrophobic-based versus hydrophilic-based fill materials) or by the physical properties of the fill material (solution versus dispersion versus solid). Hydrophobic solutions include neat oils, combinations of miscible oils, or active ingredients dissolved in oil vehicles. Hydrophobic dispersions include active ingredients dispersed or suspended in oil or in oil-wax mixtures. The latter often are termed semisolids. Hydrophilic solutions can be neat liquids, combinations of water-miscible liquids, or active ingredients dissolved in water-miscible vehicles. Hydrophilic dispersions or suspensions include active ingredients dispersed or suspended in hydrophilic vehicles such as polyethylene glycol. Solid fill materials consist of mixtures of excipients and active ingredients whose properties like hydrophilicity/hydrophobicity, polymorphism, particle size, etc., drive the dissolution behavior.

1.2 Manufacturing and Packaging Issues That Can Affect Dissolution Testing

A number of issues affect the development of a dissolution procedure and the dissolution behavior for capsules, including:

- Properties of capsule shell material
- Properties of the fill material
- Interaction between capsule shell material and fill material

Gelatin is a hygroscopic material, and its moisture content affects the properties of hard and soft gelatin capsules. Since certain excipients are known hygroscopic agents, it is particularly important to monitor the mechanical properties of gelatin capsules stored under various conditions of temperature and relative humidity. The factors that can affect the capsule properties include: moisture exchange between the shell and the fill material, which potentially can create brittleness in the gelatin shell; and chemical interactions between the fill material and gelatin, which can result in gelatin cross-linking.

The potential for aldehydic impurities and formation of degradants and degradation of the active ingredients or excipients in the formulation should be investigated during product development by means of stability studies (aldehydes contribute to cross-linking; see section *2. Cross-Linking in Gelatin Capsules*). Understanding the possible routes of aldehyde or ketone formation, and possible sources of aldehyde, helps to predict capsule behavior. The rate of cooling and drying may modify the characteristics of the active ingredient release from the matrix. The possibility of migration of the active ingredient into the capsule shell, particularly when the softgel is made of gelatin, should be investigated for its impact on dissolution testing.

Capsule shells can become reactive depending on the storage conditions. Product packaging and storage conditions are chosen to prevent adverse effects on capsule quality. The ingress of moisture and/or oxygen into the packaging and through the capsule shell, as well as the rate of ingress, can affect the final shelf life for the product. In some cases, an increase in water content of the capsule shell may produce a measurable reduction in the dissolution time because a moistened product may facilitate polymer hydration.

Due to the water content of gelatin shell (usually between 13% and 16%), this type of capsule behaves as a moisture reservoir which may affect the stability of humidity-sensitive active ingredients.

When a capsule is immersed in an aqueous medium, the water permeates the walls, the polymer becomes hydrated, and swells. When fully hydrated, the shell starts to dissolve. The amount of time it takes for water to penetrate capsule shells varies, depending on the nature of the capsules shell and other factors. This time has been reported to be approximately 40 s for gelatin capsules and about 3 min for HPMC capsules. This delay may be significant only for dissolution testing of immediate-release dosage forms, while it should have minor or no impact on dissolution testing of modified-release formulations.

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2. CROSS-LINKING IN GELATIN CAPSULES

Cross-linking involves the formation of chemical links stronger than the simple hydrogen and ionic bonding between gelatin chains and affects the thermal reversibility of the sol-gel transition of gelatin in the shell. Cross-linking can be caused by agents present in the capsule fill that react with gelatin molecules, resulting in the formation of a pellicle on the internal surface of the shell. Less often, a pellicle may form on the external surface of the shell arising from reactive agents present in, or derived from the intermediate of final packaging components.

A pellicle is a thin, water insoluble clear membrane of cross-linked protein on the inner or outer surface of the capsule that prevents the capsule fill from being released. Cross-linking is evidenced by the observation of a thin membrane or a gelatinous mass during dissolution testing because the pellicle itself may be difficult to observe.

Cross-linking can also be caused by agents or impurities present in the shell, thereby rendering the entire shell matrix insoluble under conditions that normally would dissolve the gelatin shell. One of the strongest and most common types of cross-linking involves the covalent bonding of the amine group of a lysine side chain of one gelatin molecule to a similar amine group on another molecule. This reaction is typically caused by trace amounts of reactive aldehydes. Formaldehyde, glutaraldehyde, glyoxal, and reducing sugars are the most common cross-linking agents. The covalent bonding produced with this type of cross-linking is, for all practical purposes, irreversible, and dissolution of the shell must involve the breaking of other bonds such as the enzyme-mediated breaking of the peptide bonds in the protein chains.

Gelatin that is chemically modified, e.g., by the addition of succinic acid groups to the lysine side chains, can prevent or at least hinder aldehyde-mediated cross-linking. A weaker type of cross-linking involves complexation of free carboxylic acid groups from two different gelatin molecules with trivalent metal ions such as Fe^{3+} and Al^{3+}. These cations can be found in some of the dyes used as colorants or as low-level contaminants of excipients. For higher-Bloom gelatin (see section *6.1.1 Gelatin*), which typically is considered higher quality, cross-linking occurs more readily because fewer links are needed to join greater lengths of gelatin chains.

It is extremely important to know and understand the product formulation to identify possible sources of cross-linking agents and take measures to eliminate or minimize their role in promoting cross-linking. This knowledge can help in the case of post-approval changes in the formulation and/or packaging material.

Common causes of cross-linking include the following:

- Aldehydes that are present in the active ingredient, excipients, or packaging materials (e.g., in residual solvents); or that may be formed in-situ during storage
- High humidity (leading to higher oxygen permeability)
- Substances that facilitate a cross-linking reaction
- Substances that promote decomposition of stabilizer in corn starch (hexamethylenetetramine), resulting in the formation of ammonia and formaldehyde, which cause the cross-linking reaction
- Rayon coilers that contain an aldehyde functional group (furfural)
- Polyethylene glycol may contain peroxides and aldehydes
- UV light, especially with high heat and humidity
- Aldehyde formation promoted by elevated temperatures

Dissolution testing of cross-linked capsules can result in slower release of the drug or no release at all. On rare occasions, if there are defects in the liquid-filled capsule seam, the capsule can rupture at the seam even in the presence of cross-linking in the gelatin, resulting in an early release of the capsule fill in the dissolution medium. The degree of cross-linking is not uniform within one capsule or among different capsules. As consequence, there is a higher variability in the dissolution results if the gelatin capsules are cross-linked. Enzymes can be added to the dissolution medium to overcome this problem (see *Dissolution* ⟨711⟩). Enzymes should not be used in the absence of such evidence.

3. DISSOLUTION PROCEDURE DEVELOPMENT

Design of a procedure for dissolution testing of capsules depends on the product formulation. The composition of the fill, the solubility of the active ingredient(s) in the fill, and the dispersion of the fill into the dissolution media all have an effect on the dissolution behavior of a given fill formula. For example, a formulation that contains a lipid-soluble active ingredient with hydrophobic excipients and a melting point in excess of 37° will likely not release the active ingredient into solution in aqueous media in a timeframe that is consistent with the expectations for immediate-release dosage forms. By contrast, a freely water-miscible active ingredient dissolved or dispersed in a water-soluble or water-dispersible fill formula at room temperature will be released into solution very soon after the shell ruptures and the dosage form releases the fill material into the media.

The specific design of the formulation and the target release profile should be known when developing a dissolution method for any given product. For lipid-based formulations that often are less dense than aqueous media, the release of the fill from the capsule may be driven by buoyancy. If the formulation has been designed to be self-emulsifying or self-microemulsifying, the formulation will efficiently disperse into most aqueous media. Formulations such as those that consist simply of a triglyceride with no additional co-solvent or emulsifier will rapidly float to the dissolution medium surface in the vessel. In this situation the quantitative results from a dissolution test provide the rate of drug partition from this floating layer. The apparatus selection is perhaps the most critical step for these types of capsules because the efficiency with which the capsule contents mix with dissolution media is highly influenced by the hydrodynamics of the agitation. Once the apparatus has been selected, other variables to consider in developing a dissolution test are rotation speed, dip rate or flow rate, surfactant/solubility enhancer type and concentration, and medium volume and pH (see ⟨711⟩, ⟨1092⟩, and ⟨2040⟩).

To better guide the dosage form design it may be useful to consider other characteristics of the product such as solubility of the active ingredient(s) in different media, intrinsic dissolution of the active ingredient(s) (see *Apparent Intrinsic Dissolution—*

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Dissolution Testing Procedures for Rotating Disk and Stationary Disk ⟨1087⟩), dispersibility, globule size for emulsions, micelle formation, digestion, precipitation on dilution in media, phase behavior studies, and burst tests to detect gelatin cross-linking.

The rupture test may be a useful tool in the early steps of the development and evaluation of the formulation, especially if the active ingredient belongs to Biopharmaceutics Classification System Class 1 (see *FDA Guidance for Industry—Waiver of In vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*) and is formulated in a water-miscible fill. For this purpose, rupture is considered to occur when the capsule shell is breached, exposing the fill contents. If the rupture of the capsule shell can be correlated with dissolution data, then rupture can be explored as a possible alternative to the dissolution test.

3.1 Apparatus

As is the case with other oral solid dosage forms, USP *Apparatus 1* (basket), USP *Apparatus 2* (paddles), USP *Apparatus 3* (reciprocating cylinder), and USP *Apparatus 4* (flow-through cell) are most often chosen as the dissolution apparatus for capsules (see ⟨711⟩ and ⟨2040⟩). However, several specific benefits and drawbacks must be considered in selecting and using the dissolution apparatus for capsules:

- Capsules may be filled with a material that has a specific gravity less than that of water. Therefore, the capsules may float in an aqueous dissolution medium;
- Instead of dissolving completely during the dissolution test, the capsule shell may soften and disintegrate into a sticky or waxy mass that can adhere to any point in the dissolution vessel and in different areas in different vessels, generating high variability in the results.
- The capsule fill material may form a film on the surface of the dissolution medium during the course of the test.

The capsule dissolution process involves three stages: (1) rupture of the capsule shell, (2) release and dispersion of the capsule fill material, and (3) dissolution of the active ingredient(s) in the medium. Each dissolution apparatus referred to previously can achieve these three stages, but they may cause different hydrodynamic effect upon capsules as on any other dosage form as well.

3.1.1 USP APPARATUS 1 (BASKET)

This apparatus has the advantage of enclosing the capsules, preventing them from floating freely in the medium. For certain capsules, however, baskets may not be suitable. As the capsule ruptures, the material from the capsule shell may clog the basket's mesh, and for hydrophobic fill materials the oil phase released from the capsule may not disperse into fine enough droplets in the basket to efficiently pass through the mesh. A larger-size mesh may be needed to overcome these limitations.

3.1.2 USP APPARATUS 2 (PADDLES)

This apparatus does not have a mesh that can clog, but it does not prevent the capsules from floating. In these instances, wire coils can be wound around the capsules, or commercially available sinkers can be used to encase the capsules and hold them on the bottom of the vessel, allowing the fill to become exposed to more of the medium (see *Apparatus/Agitation, Sinkers* in ⟨1092⟩). Sinkers can also be used to prevent the capsule from sticking to the vessel walls and to provide better contact with the dissolution medium even if the capsule does not float and remains at the bottom of the vessel. The shape and size of the sinker can play an important role in the dissolution profile and should be selected carefully. The swelling, that occurs when the capsule is placed in contact with the dissolution medium, should be considered when defining the size of the sinker to be used.

3.1.3 USP APPARATUS 3 (RECIPROCATING CYLINDER)

This apparatus, like *Apparatus 1*, encloses the capsules. The mesh at the bottom of the cylinder, however, may become clogged with undissolved shell. Changing the mesh size may alleviate this problem. The different mechanism of agitation of *Apparatus 3* provides very different hydrodynamics compared with *Apparatus 1* and *2*. This characteristic may assist in dispersing hydrophobic droplets, avoiding the formation of layers and some problems caused by the buoyancy of the capsule. In the case of *Apparatus 3*, a change to different media during the course of the dissolution experiment is possible. This allows dissolution profiles to be determined at different pH values, which is useful for targeted-action or modified-release dosage forms. For cases where the active ingredient has been dispersed but not dissolved, this apparatus may not be a good choice since significant sample loss will occur when moving the cylinder from one tube to the other. However, USP *Apparatus 3* has a tendency to generate foam when surfactants are added to the media. In this situation, an appropriate antifoaming agent may be added to the medium.

An alternative to *Apparatus 3* is the disintegration apparatus described in *Disintegration* ⟨701⟩. The use of disks should be avoided because of clogging. If the discs are not used, the capsule will float and may not be uniformly wetted. This apparatus offers more turbulent conditions that could be useful in the dispersion of hydrophobic filling, avoiding the formation of layers and counteracting the buoyancy of the capsule.

3.1.4 USP APPARATUS 4 (FLOW-THROUGH CELL)

This apparatus also encloses the capsules and has a filter. Several types of cells are available depending upon the application. The use of the flow-through cell designed for lipid-filled softgels may be useful for certain types of formulations (see *Figure 2* in ⟨2040⟩). Use of *Apparatus 4* also makes it possible to change to different media and to alter the flow during the course of the dissolution experiment. In addition, this equipment can be set up for low or high volumes of media that can be used with low-strength products and with poorly soluble active ingredient(s), respectively.

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Other apparatus may be considered with proper justification, with each candidate apparatus evaluated for variations in media composition, media volume, agitation or flow rate, and other test parameters to determine what effect each has on the dissolution performance of the product.

3.2 Medium

General recommendations for the selection of appropriate dissolution media can be found in chapters ⟨1092⟩ and ⟨2040⟩, *FDA Guidance for Industry—Dissolution Testing of Immediate-Release Solid Oral Dosage Forms* and *FDA Guidance for Industry—Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In vitro/In vivo Correlations*.

Characteristics of the dissolution medium that may affect the opening or rupture of the capsule shell, the release and dispersion of the capsule fill material, and the dissolution of the active ingredient(s) are discussed in the following sections.

3.2.1 ACHIEVING SINK CONDITIONS

As in the development of any effective dissolution procedure, the medium preferably, but not necessarily, should provide sink conditions for the active ingredient(s) in an environment that ensures suitable stability and, preferably, is physiologically relevant for the product (see ⟨1092⟩).

If the fill is water-soluble or, at least, readily dispersible in an aqueous medium, and if the active ingredient itself is also soluble, sink conditions may be achieved in a manner comparable to that for any other solid oral dosage form. Liquid-filled capsules, however, may contain either a matrix or active ingredient (or both) that are hydrophobic or water-insoluble. In this case, a medium with surfactants may be needed (see below). The use of organic co-solvents to improve sink conditions is discouraged and should be employed only as a last resort and at a minimum amount with appropriate justification. In practice, the presence of organic solvents in the medium may inhibit the dissolution of the shell.

In addition to dispersing/dissolving the matrix and active ingredient(s), the medium must neither interfere with the activity of any enzymes used nor negatively interact with the capsule shell or formulation. Developing a suitable medium for hydrophobic systems therefore may require considerable experimentation. For example, using testing from ⟨711⟩, the addition of enzymes is required when the gelatin capsule fails the test due to pellicle formation. When certain surfactants are used (e.g., sodium lauryl sulfate), the enzyme may be deactivated, depending on the type and concentration of surfactant used. In this case, a pretreatment period with the proteolytic enzyme followed by a later addition of the surfactant to the medium may be considered. The time of this pre-treatment should be as short as possible. In most cases, it does not exceed 15 min. The pre-treatment period is included in the total time of the dissolution testing.

To establish a suitable medium, several different dissolution media should be evaluated to identify the one that achieves appropriate sink conditions with the lowest quantity of solubilizing/dispersing agent. The effect of pH, ionic strength, buffer counter-ion and/or co-solvent on active ingredient solubility and enzyme activity, particularly for hydrophobic active ingredients, in addition to the relative partitioning of the active ingredient between the matrix and medium, must also be evaluated.

3.2.2 USE OF SURFACTANTS/DISPERSING AGENTS/SOLUBILITY ENHANCERS

Surfactants, dispersing agents, or solubility enhancers may be used in the dissolution medium when the capsule fill, the active ingredient, or both, are hydrophobic or water-insoluble. They may also be used if the media described in ⟨1092⟩ and in the FDA guidance are ineffective in dispersing the capsule fill or in achieving proper sink conditions for the active ingredient.

Surfactants, dispersing agents, and solubility enhancers include:

- Anionic:
  - Sodium dodecyl sulfate (SDS, or sodium lauryl sulfate, or SLS)
  - Bile salts (sodium deoxycholate, sodium cholate)
- Cationic:
  - Cetyltrimethylammonium bromide (CTAB)
  - Hexadecyltrimethylammonium bromide (HTAB)
  - Methylbenzethonium chloride (Hyamine™)
- Nonionic:
  - Polysorbates (Tween™)
  - Polyoxyethylene sorbitan esters
  - Octoxynol (Triton X100™)
  - *N,N*-dimethyldodecylamide-*N*-oxide
  - Brij 721
  - Polyoxyl castor oil (Cremophor™)
  - Nonylphenol ethoxylate (Tergitol™)
  - Cyclodextrins
  - Polyoxyl 10 lauryl ether
- Zwitterion
  - Lauryl dimethyl amine oxide (dodecyldimethylamine oxide, DDAO)
  - Lecithin

Although useful as solubilizing agents, surfactants should be used cautiously because they can interact with the gelatin in the capsule shell and can hinder disintegration or dissolution. They can also inhibit enzymes that may be used to hydrolyze the gelatin shell and/or the fill.

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One of the most common anionic surfactants used for dissolution testing of tablets, SLS, exhibits both of these adverse effects, particularly at lower pH (e.g., in simulated gastric fluid). In addition, SLS forms insoluble precipitates in the presence of potassium ions. SLS is one of the surfactants less compatible with enzymes. Only a very high quality grade of SLS should be employed in dissolution media because of the potential interference of its impurities in the quantitation step in dissolution testing. Therefore, the use of SLS should be considered only in the absence of other alternatives.

Other anionic or cationic surfactants have also been shown to affect gelatin solubility, and the extent of these interactions should be considered in the selection of the dissolution medium. Cationic surfactants should be avoided in formulations that contain fatty acids because the combination potentially forms insoluble precipitates.

3.2.3 USE OF ENZYMES

Proteolytic enzymes such as pepsin (at low pH) or pancreatin (at pH ≥6.8) may be used when the gelatin capsule shell does not dissolve in ordinary aqueous media because of cross-linking, as previously discussed. Pancreatin has the advantage of also possessing lipase activity, which makes it useful when the capsule fill material is a triglyceride.

Tests should be carried out to ensure that the enzymes used in the dissolution testing do not adversely interact with the formulation. The dissolution medium should also be optimized (e.g., by adjusting the pH, ionic strength, etc.) so that it preserves enzymatic activity while maintaining sink conditions for the active ingredient. If surfactants are also used, their potential adverse effect on enzyme activity must be evaluated.

3.2.4 pH

In addition to establishing a drug pH-solubility profile during the dissolution development, the following specific issues should also be evaluated:

- The effect of media pH on the swelling or dissolution of the capsule based on the type of gelatin used in the product: either Type A (for which the medium pH typically is 7–9) or Type B (for which the medium pH typically is 4.7–5.4)
- The need for enzymes may influence the selection of a specific pH range in order to be adequate for the active ingredient solubility and stability and to minimize effects on enzyme activity.

3.3 Cross-Linking in Gelatin Capsules

As previously discussed, cross-linking results in the formation of pellicles on the internal or external surface of the shell. Depending on the extent of the cross-linking and the structural integrity of the shell, this pellicle may delay or prevent the release of the fill and the dissolution of active ingredient(s) during the test.

Pellicles often are difficult to dissolve and typically require the use of proteolytic enzymes in the medium to achieve dissolution.

Gelatin also forms ionic cross-links (salt bridges), typically between carboxylate (anionic) and ammonium (cationic) side chains of the amino acids comprising the polypeptide chain. Although they can inhibit gelatin dissolution, ionic cross-links are much weaker than covalent cross-links and may be disrupted by changing the ionic strength or pH of the medium or by using an enzyme.

3.3.1 FORCED CROSS-LINKING

During product and dissolution method development, forced formation of cross-linking in the gelatin capsule may be useful to establish the type and amount of enzyme that will be used in the test, and to better understand the behavior of the formulation in the dissolution medium. This can be achieved by spiking excipients with known amounts of formaldehyde or other cross-linking agents, or exposing the capsules to high humidity.

3.4 Sampling

Establishing the proper sampling technique and location for capsules follows the procedures described in chapters ⟨711⟩ and ⟨1092⟩. In the case of hydrophobic liquid filled capsules, the fill material typically forms a film on the surface of the dissolution medium during the course of the test, sampling must be performed in such a way that the cannula penetrates the oily layer without being clogged. In addition to standard validation and compatibility studies, care must be taken when a filter is used to ensure that it does not become clogged with oil or undissolved capsule shell material when the sample is taken. Similar considerations apply to automated sampling equipment because filters and transfer lines may become obstructed during sampling. To address this issue, the use of surfactants and/or enzymes in dissolution media may be needed to better solubilize the capsule shell and fill. Another point to be considered in the case of automated sampling equipment is the probe. If it remains inside the medium during the test, it could perturb the oily layer and possibly influence the hydrodynamics and change the dissolution profile. An alternative approach is to remove the probe and to introduce it just at the time of the sampling. The sampling method should be validated for each product.

3.5 Quantitation

Like dissolution samples from other oral solid dosage forms, dissolution samples from capsules can be quantitated using chromatographic, spectrophotometric, tandem chromatography-mass spectrometry, and other techniques after adequate method development and validation (see ⟨1092⟩ and where special considerations are described as follows under section *4. Method Validation*).

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4. METHOD VALIDATION

In addition to the general method validation parameters discussed in ⟨1092⟩, the following performance characteristics may be evaluated:

- Effect of pH and ionic strength on drug solubility and on enzyme activity, if enzymes are used
- For hydrophobic active ingredient(s), the relative partitioning of the active ingredient(s) between the matrix and the medium, as well as the potential for an adverse effect on release into the aqueous medium
- For chromatographic procedures, the potential adverse effect of the surfactant, if used, on the chromatographic separation; as part of the robustness study, evaluation of different concentrations of surfactant, different surfactant types, interaction with buffer salts, etc.
- For spectrophotometric procedures, absorbance from the potential contribution of the capsule shell should be evaluated
- Potential adverse effects of the surfactant or enzyme, if present, on the lifetime of the chromatographic column
- Qualification of the sampling procedure to prevent clogging of the cannula, transfer tubing, or filters
- Validation of the second tier dissolution test
  - pre-soaking time
  - compatibility of any surfactants present with the enzymes
  - verification that the conditions of the second tier test do not affect the dissolution profile when compared to the first tier test

5. SUGGESTIONS FOR STARTING POINTS

Based on the considerations discussed above, possible starting points for establishing a dissolution test for capsules based on the solubility characteristics of the fill and the active ingredient(s) are the following:

Possible formulations:

1. Hydrophilic fill/active ingredient is soluble in aqueous media
2. Hydrophobic fill/active ingredient is poorly soluble in aqueous media
3. Hydrophilic fill/active ingredient is poorly soluble in aqueous media
4. Hydrophobic fill/active ingredient is soluble in aqueous media

5.1 Medium

Evaluate the solubility of the active ingredient(s) in aqueous media within a pH range of 1.0 to 7.2–7.5.

5.2 Medium Additives

Enzymes such as pepsin (at pH <4.0) or pancreatin (at pH ≥6.8) may be used if the product shows evidence of cross-linking in the gelatin capsule. If the product is a formulation such as *2* or *3* above, evaluate the use of surfactants and possible interaction with enzymes.

5.3 Apparatus

USP *Apparatus 1* or *2*. If the capsule is filled with low-specific-gravity liquid and has a tendency to float, sinkers should be used. If the product is a formulation such as *2* or *4* above, consider *Apparatus 3* as an option.

5.4 Agitation Speed

Agitation speed typically is 50–100 rpm for baskets and 50–75 rpm for paddles. Higher speeds should be justified.

5.5 Time Points

A dissolution profile should be established during the development phase to identify when the rate of active ingredient release has leveled off.

6. CRITICAL QUALITY ATTRIBUTES

6.1 Shell Composition

Knowledge of the composition of the shell (polymer, plasticizers, water content, etc.), as discussed at the beginning of this chapter, is an important aspect and helps determine critical quality attributes. Other important aspects of understanding the critical quality attributes are the composition and properties of the gel mass and finished shell. In its simplest form, the shell of a capsule is prepared from a molten gel mass, which in the case of hardgels comprises gelatin or other polymers and water and in the case of softgels the shell is comprised of gelatin and a plasticizer dissolved in an aqueous vehicle. The ratio of polymer

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to plasticizer varies depending on the desired performance traits of the shell, the size of the shell, and the composition of the fill material. Other minor components added to the gel mass may include colorants, flavors, stabilizers, buffers, and opacifiers. The physical characteristics and quality of these minor components are central to the design of high-quality and robust formulations. The composition of hardgels usually does not vary with capsule size.

6.1.1 GELATIN

Gelatin capsule manufacturing process: For hardgels, there are two separate manufacturing steps: (1) manufacturing the empty shells and (2) the filling process. Capsule shells are produced, packaged, and shipped to the dosage form manufacturers. Capsules are then filled and sealed. Most modern capsule-filling machines are designed to allow accurate filling of powders, granules, pellets, tablets, and combinations of these and many can be modified to allow hot or cold liquid to be filled into hardgels. An essential part of a liquid-filling operation is the ability to effectively seal the capsule. This sealing process is a critical process parameter, and detailed knowledge of all the aspects of this process is important.

For softgels, the formation of the capsule, the filling process (encapsulation process), and sealing of the capsules occur simultaneously. During the encapsulation process, it is important to monitor the shell thickness, seam quality, capsule weight, and the fill weight using statistical process controls.

Chemistry of gelatin: Understanding the chemical nature of gelatin is another important component of the critical attributes of the formulation. Gelatin is graded primarily on the strength of the gel. Depending on the process and the tissue source, noticeable differences in strength are apparent among suppliers and even between lots from the same supplier. Consequently, controlling the strength of the gelatin from batch to batch, measured as Bloom strength, is key to obtaining a consistently performing product. Bloom strength is a measure of the strength of the gel prepared at a set concentration of gelatin in water under controlled conditions and is a function of the molecular weight of the gelatin, the concentration of the gelatin in the gel, and the pH of the gel. It is a measure of the resultant gel's resistance to compression and is reported in Bloom-grams or grams. Bloom strength increases when the gelatin concentration in the gel increases, when the average molecular weight of the gelatin increases, and when the pH of the gel approaches neutrality (from either direction). In addition, as Bloom strength increases, the cost of gelatin increases and the rate of gel dissolution decreases. Bloom strength also has an effect on the clarity and color of liquid-filled capsules. For gelatin with higher Bloom strength, less gelatin is needed to produce a suitable shell, which results in a clearer shell and a reduced need for colorants and dyes in order to produce the desired hue. Although gelatin can be purchased with Bloom strengths ranging from 50 to 300, most gelatins used in the manufacture of liquid-filled capsules have a Bloom strength of about 150–200 for softgels and 220–280 for hardgels. Gelatin manufacturers commonly blend different sublots of gelatin to meet Bloom requirements. These important characteristics of gelatin must be taken into account when characterizing the product under development.

6.1.2 CHEMISTRY OF OTHER POLYMERS

Suitable consideration should be given to the other polymers used to manufacture capsule shells. Iota and kappa carrageenans, modified corn, potato, and pea starches, and modified celluloses, together with plasticizers are also used for the preparation of capsule shells. Carrageenans are polysaccharides extracted from sea weeds. They possess the required gelling characteristics similar to gelatin. However, kappa carrageenan produces a brittle gel, and iota carrageenan a soft and elastic gel. Carrageenans are usually combined with modified starches and plasticizers to form the capsule shell. Modified celluloses, such as hypromellose (HPMC), are also used for the manufacturing of hardgels. Compared to gelatin shell, the shell prepared using the polymers mentioned above will have some advantages, such as non-crosslinking, being able to handle wider pH ranges, and tolerance of high fill temperature.

6.1.3 PROCESSING AIDS

Processing aids include gelatin ribbon lubricants in the softgel manufacturing process. Commonly used ribbon lubricants include mineral oil and medium chain triglyceride. Since ribbon lubricants are applied to both the inner and outer surfaces of the capsule shell, the evaluation of their potential adverse effects is focused on the capsule itself and primarily on gelatin cross-linkage.

6.2 Stability and Storage Conditions

Knowledge of the stability and reactivity of gelatin or other polymers is essential to anticipate their influence on final product quality. At room temperature, the capsule shells as supplied are relatively effective in protecting the fill from oxygen and its effects, and when an opacifier such as titanium dioxide is added to the shell, it can prevent photodegradation of the fill. In addition, the low water activity of the shell does not promote microbial growth. For bacteria, yeasts, and molds to grow a higher water activity of at least 80 aw is required; the water activity of capsule shells typically is less than 0.40 aw.

Storage conditions of the final product range from refrigeration to standard room temperature. Products should be stored according to the label directions. Brief excursions outside of these conditions should be evaluated to determine their influence on the final product quality. Storage conditions and duration for the empty capsule shells should also be considered.

6.3 Formulation Development and Manufacturing for Liquid-Filled Capsules

It is important to understand the properties of the active ingredient(s) during formulation development.

Formulations developed using liquid-filling technology may be applicable when the active ingredients (1) display poor solubility in aqueous systems, (2) short half-life requiring frequent dosing, (3) low melting point, (4) low dose/high potency where containment is important, (5) requirement for taste or odor masking and (6) critical chemical or physical stability as in the case of highly hygroscopic active ingredients. Other factors to consider during formulation development include:

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- Compatibility and stability of excipients and active ingredient(s) over time
- Temperature-dependent solubility of the active ingredient(s) in the lipid
- Aging/polymorphic characteristics of the lipid
- Adequate characterization of the saturation/supersaturation status of the active ingredient(s) in the lipid formulation in order to avoid precipitation of the active ingredient(s). Optimally, saturation status should be determined when the formulation is at equilibrium with the shell.
- Stability in solution under stress conditions
- Aldehyde formation and degradation of active ingredient(s)
- Influence, if any, of the rate of cooling on the structure of certain excipients, which may modify the release characteristics of the active ingredient(s)

Important factors to consider during a liquid-filling operation are temperature and viscosity of the fill material and, in the case of a dispersion or suspension, the particle size of the dispersed active ingredient(s). In principle, any excipient found to be compatible with the shell can be used, but in a manufacturing environment the viscosity of the fill material is important. Excipients that are solid at room temperature but melt at temperatures up to 70° may be suitable (depending on the shell polymer) for formulating active ingredient(s) provided those excipients yield the desired in vivo performance.

Both shell and fill excipients should be controlled for levels of known cross-linking agents such as formaldehyde and reducing sugars.

Imperfections in the shell and/or in the seam may affect dissolution. They may also give rise to leaking of the capsules contents.

The appropriate in-process controls should be in place to monitor and reduce the lot-to-lot variability.

⟨1097⟩ BULK POWDER SAMPLING PROCEDURES

INTRODUCTION

The goals of this chapter are to provide guidance on bulk powder sampling procedures, identify important bulk powder sampling concepts, and collect a knowledge base of useful practices and considerations that can lead to the ideal physical sampling of bulk powder materials. The terminology used here is well established in the field of material sampling (see *Appendix 3*, for instance reference *7*). Sampling is undertaken as part of an estimation process. The parameter of primary interest here is the mean level of some analyte in the bulk powder as a whole.

The purpose of a sampling plan is to obtain a representative sample of a population so that reliable inferences about the population sampled can be drawn to a certain level or degree of confidence. Acquiring a representative sample from a lot is critical because without a representative sample all further analyses and data interpretations about the lot are in doubt. An ideal sampling process is a process in which every particle or at least every equal-size portion of the population has an equal probability of being chosen in the sample. In addition, sampling procedures should be reproducible, i.e., if the sampling protocol were repeated, a high probability should exist of obtaining similar results. Also, the integrity of the sample should be preserved during and after sampling. The details of how to sample depend on a variety of factors. For example, criteria for sampling to evaluate particle segregation may differ from criteria for evaluating moisture content or identification.

Because of the propensity of a powder to segregate, heterogeneous powder systems can make it difficult to obtain an ideal sample. Thus, to extract representative samples requires careful development of a sampling plan that accounts for and mitigates the segregation tendencies of a particular powder system. Developing a general guidance for bulk powder sampling is challenging because every situation is different, and therefore different approaches must be used to deal with each situation. Thus, the goal of this general information chapter is to outline recommended steps for developing a sampling scheme or plan for a particular system that is consistent with good sampling practices.

The primary difficulty in acquiring a representative sample is that the size of the sample for measurement, typically a few milligrams to grams, must be withdrawn from a large population on the order of hundreds to thousands of kilograms. The few milligrams analyzed in a laboratory must be taken from a large population of particles in a warehouse in such a manner that the measurement sample is representative of all the particles in the lot. Any bias or error in the sampling process will cause all future inferences to be in error. Over the years methods have been developed and refined to attempt to ensure that the measurement sample is representative of the whole population. A typical strategy is shown in *Figure 1*. The strategy is to sample in stages, starting with the initial gross or primary sample withdrawn directly from the received containers. In the laboratory, the gross sample must be reduced in size until it is the appropriate size for measurement. This should be done in a manner that minimizes the introduction of sampling errors. The key to reducing the sampling error is to ensure that every particle of the population has an equal probability of being included in the sample. However, because of segregation or the nonrandom nature of powders, many obstacles can cause bias and contribute to sampling errors. Following the flow chart in *Figure 1* and the steps outlined in subsequent discussions will help to minimize sampling errors.

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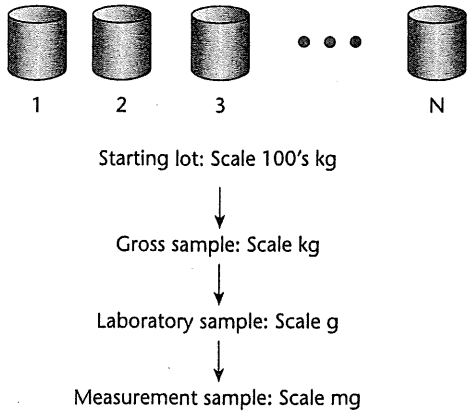

Figure 1. Overall sampling strategy for reducing the sample size from the hundreds of kg scale to the mg scale.

To acquire a representative sample, a suitable sampling plan must be developed and implemented. A good sampling plan includes: (1) population determination and sample size selection, (2) a sample collection procedure and a method for sample size reduction, and (3) summary calculations that demonstrate that the sampling plan will yield samples that accurately characterize the population to within a stated level of acceptance. In addition, an infrastructure is needed to maintain the integrity of the samples and sampled materials.

This chapter begins with a brief introduction to sampling theory and terminology. The technical content of the chapter requires a basic scientific understanding of physical particle characteristics (e.g., mass, density, shape, and size) and statistics (e.g., acceptance sampling and binomial distribution).

SAMPLING THEORY AND TERMINOLOGY

Fundamental Sample Size (Sample Mass)

Sample size is considered from two perspectives: (1) the mass of the sample intended to represent the entire population, sometimes termed the composite sample, and (2) the number of samples taken with a mass sufficient to independently evaluate, compare, or provide confidence to ensure the reproducibility of the composite or the uniformity of the population. The key to obtaining an ideal sample is to understand and account for the degree of heterogeneity of the characteristic being evaluated in the system under study. For example, heterogeneity of a particle system arises from two sources: the intrinsic, constitutive, or compositional heterogeneity and the spatial distribution heterogeneity. The intrinsic heterogeneity of the powder system reflects the fundamental differences in the individual particles. Statistical heterogeneity (differences between individuals), or variance, is expected to maintain assumed properties. For a normal population the general expression for a statistical sample size suggests that the number of independent samples is proportional to the square of the normal quantile at the desired confidence level (Z) and the population variance (σ^2) and is inversely proportional to the square of the minimum detectable difference required (δ), as shown in equation 1:

$$n \propto \frac{Z^2\sigma^2}{\delta^2} \qquad (1)$$

In order to apply the normal theory sample size equation to sample mass with a discrete number of particles, consideration for material characteristics is needed. For a heterogeneous bulk material, such as a bulk powder, the sample mass required to ensure adequate representation of the intrinsic or fundamental population heterogeneity or variation is determined by the size, shape, and density of the particles. The total sampling error (TSE) measures the difference between the analyte concentration estimated in the sample (a_{sample}) and the mean analyte concentration in the lot (a_{lot}) relative to the mean analyte concentration in the lot (a_{lot}), as shown in equation 2:

$$TSE = \frac{a_{sample} - a_{lot}}{a_{lot}} \qquad (2)$$

When ideal sampling is employed, the TSE is reduced to a fundamental sampling error, limited only by the intrinsic heterogeneity of the material. The relative variance of the fundamental sampling error (S_{fse}^2) has been empirically estimated in particle size applications by characterizing the critical particle mass, heterogeneity, size (diameter), shape, density, and weights of the material. Empirical estimates require a thorough and complete knowledge of the material and process. Established material characterization and methods are critical aspects of avoiding unacceptable estimates. As shown in equation 3:

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$$S_{fse}^2 \propto f_{shape}\, g_{CF}\, c_{max}\, l d_{max}^3 \left(\frac{1}{m_{sample}} - \frac{1}{m_{lot}} \right) \quad (3)$$

where f_{shape} is a measure of cubicity or shape factor of the analyte particles; g_{CF}, the granulometric factor, is an empirical correction factor of differences in particle size; c_{max} is the compositional maximum heterogeneity and is calculated as if the material consists of the analyte particles and everything else; l, the liberation factor, is an empirical factor representing the proportion of critical content particles separated from the non-analyte containing particles of the lot; d_{max} is the particle diameter [e.g., the maximum diameter or the diameter (cm) of the size of the opening of a screen retaining 5% by weight of the lot to be sampled]; m_{sample} is the mass of the sample; and m_{lot} is the mass of the lot being sampled. [NOTE—A liberation factor is needed when the analyte does not appear as separate particles. A high liberation value (1.0) suggests heterogeneity of particles. A low liberation value (0.05) suggests very homogeneous particles. See *Appendix 1* for examples of potential applications of equation 3 in the estimation of the fundamental sample mass needed to account for constitutional heterogeneity of the powder mixture.] Use of equation 3 requires prior estimates of f_{shape}, g_{CF}, c_{max}, l, and d_{max}.

Segregation Error

Distribution heterogeneity is the difference between samples or groups of particles spatially or temporally. For example, small particles are located preferentially in the lower portion of a powder bed. This type of situation can arise as a result of powder bed segregation and is common in some particle systems with a broad particle size distribution. In other words, smaller particles may not be randomly distributed throughout the lot. This spatial heterogeneity introduces variation in the sample and is a source of variation that contributes to the total variation. Together, fundamental and segregation error give rise to sampling error, which dictates how variable the samples will be, how large the sample size and numbers of samples should be (e.g., 10 containers, sampled at top and bottom, with sample sizes of 50 g each), and how hard it will be to obtain a representative sample.

Minimizing the effects of segregation error during lot material characterization while still ensuring a representative sample mass requires collecting many small samples that average out the variation of the segregation error. This assumes one is interested in estimating the overall average, not characterizing lot heterogeneity. Segregation error is difficult to control because segregation may be the result of changes in particle size, shape, and density, as well as inputs into the determination of sample mass. Minimizing the effects of segregation error when reducing the primary sample size requires adequate physical mixing or randomization of the primary samples before analysis, thus providing equal selection probability.

Total Sampling Method Error

Intrinsic or compositional heterogeneity is a function of the powder system and represents the true characteristics of the material (e.g., equation 3). Thus, intrinsic heterogeneity is often the minimal variance a system can have. The difference between the true state of the system and what is actually measured when ideal sampling is employed is called the fundamental error (equation 2). The relative variance of TSE (S^2_{Total}) is represented in equation 4 as the sum of the relative variances of all error components:

$$S^2_{total} = S^2_{fundamental} + S^2_{segregation} + S^2_{extraction} + S^2_{delimitation} + S^2_{preparation} + S^2_{trends,\ shifts} + S^2_{cycles} + S^2_{analytical\ method} \quad (4)$$

The S^2_{Total} can be reduced by employing ideal sampling. Ideal sampling will limit or adjust for the effects of error contributed by particle segregation, extraction error created by the sampling device, delimitation error created by not considering the three-dimensional nature of the bulk material, and sample handling errors such as product degradation. The total variation is the sum of these sources of error, illustrated in equation 4 as independent, additive components. To the end of reducing these errors, an important goal of material characterization by sampling is the determination of the relevant errors within the bulk sample. Knowing the source of the error helps determine how to best minimize these errors.

Fundamental error arises from the intrinsic heterogeneity of particles within a sample of the material population. Reducing fundamental error requires changing the intrinsic characteristics of the material, such as reducing the particle size by milling or grinding. Segregation error is the spatial distributional difference of particles across the population. This type of error can be minimized by mixing or randomization of the particles being selected. Segregation error is affected by the characteristics of fundamental error. Additionally, for the determination of both fundamental and segregation error, it is assumed that mechanical sampling is carried out correctly and is not invasive, i.e., that mechanical sampling does not alter the characteristics being measured and provides a true representation. In instances where sampling of the bulk material does not provide unbiased representation or is so invasive that it alters material characteristics, then, in order to obtain noninvasive, unbiased samples, operators may need to change sampling from a bulk form to a stream form of processing, either upstream or downstream from the sample point (see *Appendix 2*). The mechanical sampler may need to mix the sample sufficiently to facilitate random sampling with equal probability of selection in order to obtain an adequate representation of the entire bulk lot. The process may also require mixing or sampling from a location in the process that will provide a random sample from material that is susceptible to segregation.

Extraction, delimitation, and handling errors occur as a result of the mechanical sampler and sample handling prior to analysis, which also are affected by fundamental error. Trends, shifts, and cycles are temporal sources of error that affect total error. The analytical error of the method of analysis contributes to the overall error of the reported result. In addition to obtaining representative subsamples from the bulk material, the method must also obtain a representative subsample from the particulate laboratory sample before analysis.

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Sampling Strategy

A typical sampling strategy consists of two basic steps: (1) the primary or gross sample, followed by (2) the secondary sample, which reduces the primary sample to a size that is suitable for laboratory measurement. In short, the goal is to select from the lot a quantity of material suitable for measurement without significantly changing the attribute for which one is sampling. In parallel with the sample size reduction, sample size calculations must be done in such a way that the sampling strategy has sufficient statistical power to determine whether the attributes of interest lie within the specification ranges with a reasonable degree of certainty. Each step must be done correctly, or the sampling strategy as a whole will not provide a sample that is representative of the original population.

To successfully withdraw a sample from a bulk container that is representative of the population, one needs to have an idea of the population's heterogeneity, i.e., how segregated or stratified the system is. Knowing what factors can accentuate segregation and knowing the patterns of segregation that are likely will help one to account for segregation in a powder bed and to take better samples. Many factors can affect the degree of powder bed segregation. For segregation to occur, sufficient energy needs to be put into the powder bed to induce motion between particles. When a sufficient amount of energy is supplied, segregation can occur via three modes: percolation (in the powder bed), rolling (on the free surfaces of a powder bed), and free flight (when the powder bed is fluidized). These modes are illustrated in *Figure 2*.

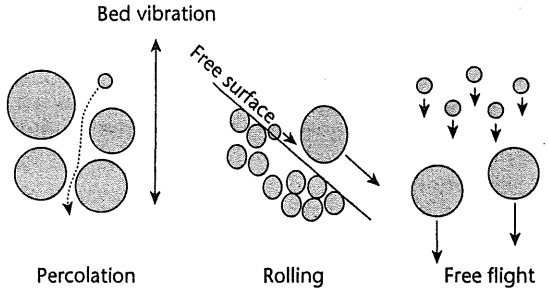

Figure 2. Illustration of the three modes of particle segregation: percolation, rolling, and free flight.

Within the powder bed, segregation can occur by means of percolation, also called sifting segregation, as well as through the movement of coarse particles to the top via vibration. During sifting segregation, smaller particles acting under the influence of gravity can more easily migrate downward into the void spaces between larger particles when the particle bed is perturbed. The net effect of these movements is that the smaller particles percolate down into the powder bed, resulting in the top of the powder bed having a higher proportion of larger particles. A common example of sifting segregation is unpopped corn kernels that are found at the bottom of a bag of popped popcorn.

For free surfaces, rolling segregation can occur any time that particles can roll down a free surface. In other words, segregation can occur on any non-level surface that allows the relative movement of particles. When particles roll down these free surfaces, larger particles tend to tumble farther down the surface than the smaller particles (see *Figure 3*). For example, if a conical heap or pile is formed in the middle of a hopper during loading, larger particles are more likely to roll farther down the heap, toward the outer edge of the hopper. This creates a situation in which the smaller particles tend to be in the center of the hopper, and the larger particles accumulate toward the outer wall of the hopper. The formation of these free surfaces can be a major factor in segregation.

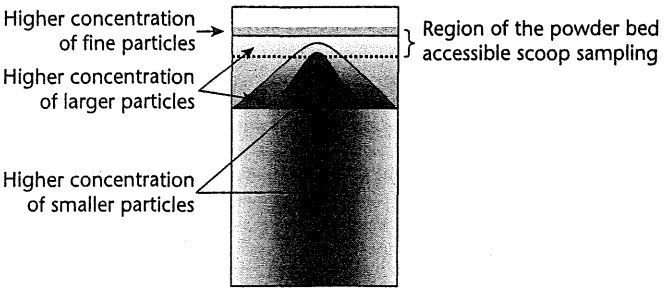

Figure 3. Example of extensive powder segregation within a drum.

When powder beds are fluidized, a large amount of air is incorporated into the powder bed and, when this air is moving, the air velocity may exceed the terminal velocity of the smaller particles. When this happens, the fine particles are suspended in the air stream while the coarse particles settle out. The fine particles eventually settle on top of the powder bed, forming a top layer that has a higher concentration of fine particles. This type of segregation, sometimes called elutriation segregation, can occur when a powder is discharged from a hopper, or is poured into the top of a hopper, and a large volume of air is displaced.

In summary, for a highly segregating system, the powder bed could have a particle distribution similar to that shown in *Figure 3*, where, as a result of elutriation segregation, a layer of fine particles on the top overlies larger particles deposited by

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percolation segregation, and a radial distribution of larger particles appears toward the outer wall as a result of rolling segregation.

In general, the primary factors that affect segregation are particle size and size distribution, density, and shape and shape distribution. Of secondary importance are surface roughness, surface coefficient of friction, moisture content, and container shape and design. Particle size is the most important single factor, and subtle differences in particle size can cause measurable segregation. If the attribute of interest is associated with particle size, then this attribute will segregate along with the different particle sizes. For example, if a manufacturer makes a granulation in which the larger particles contain more drug than the smaller particles, then drug content can be very prone to segregation—i.e., drug content will show segregation patterns similar to those associated with particle size segregation.

Segregation can notably increase sampling error because it decreases the probability that certain particle types will be in the sample. In addition, the powder bed may already be segregated when material is received, and poor sample handling can also cause segregation. To avoid further segregation during sample handling, the operator should avoid situations that promote segregation, such as the following: pouring where the powder forms a sloping surface, pouring into the core of a hopper, vibrations, shaking, and stirring (unless done to promote mixing). In addition, the use of mass flow hoppers reduces segregation.

Two basic strategies help promote ideal sampling: (1) use of a sampling thief and (2) sampling from a moving powder stream.

A sampling thief is a long spearlike probe that can be inserted into the powder bed and, once inserted, can collect powder samples from points adjacent to the spear. With a sampling thief, particles from almost any point in the powder bed can be included in the sample. The second method relies on fundamental principles of sampling, namely that (1) a powder should always be sampled when in motion, and (2) the whole stream of powder should be sampled for many short periods rather than sampling a part of the stream for a longer period.

For example, if the container to be sampled is emptied onto a conveyer belt, all the material will pass by a single point that can be sampled. Thus, no matter how segregated the system is, the collection of the powder at random time points ensures that every particle has an equal probability of being included in the sample. The second fundamental principle accounts for material segregation on the conveyer belt: by collecting the entire stream, one gets a cross section of all the particles, no matter how much segregation occurs on the conveyer belt.

Many methods are available for obtaining a sample from a powder system. Unfortunately, many of these methods involve setting the powder bed in motion or performing in-process sampling. Because of concerns about cross-contamination and containment of potentially toxic materials, most of these methods are impractical for the bulk sampling required for compliance with current Good Manufacturing Practices (cGMPs). Hence, most of the sampling done in the pharmaceutical industry is static sampling, done by either (1) scoop or grab sampling or (2) stratified sampling, typically employing a sampling thief. The choice of method is dictated by the distribution of the attribute being sampled in the container, as discussed below.

GENERAL SAMPLE COLLECTION: CONSIDERATIONS AND TOOLS

Types of Systems and General Considerations

HOMOGENEOUS SYSTEMS

For powder systems where the attribute of interest is uniformly distributed throughout the container—so that any sample is an unbiased representation of the entire container, lot, or population of interest—scoop sampling is adequate. Scoop sampling is a straightforward procedure in which the operator, after selecting representative containers for sampling, opens a container, scoops out a sufficient amount of material from the top of the powder bed, and then seals the container. If a thin layer of material on top of the powder bed is suspected of being different from the bulk, samples should be taken from a point below this top layer. For example, in cases of elutriation segregation, a thin layer of fine particles may lie on top of the powder bed, and the operator should dig down into the powder bed to avoid sampling from this layer. The scoop should be large enough that no material is lost during handling, because lost material may result in sample bias. In other words, one should avoid the use of a heaping scoop from which material can roll off the sides. The advantages of scoop sampling are convenience and cost, and, for highly potent materials, low-cost disposable scoops that can be used to minimize cross-contamination.

HETEROGENEOUS SYSTEMS

If the attribute of interest is spatially distributed in a heterogeneous manner throughout the sample, then scoop sampling is prone to potentially significant errors. Scoop sampling is a non-probabilistic method because only the most accessible fraction of the container is sampled. Obviously, only the material in the top layer can be reached with a scoop. For example, a sample from the top outer edge of the drum shown in *Figure 3* could be biased because, in this example, the larger particles are preferentially distributed toward the top and outer edges of the drum. Hence the smaller particles have a lower probability of appearing in the sample. As a result, the smaller particles will be underrepresented in the sample, and any analysis of particle size will not reflect the true particle size distribution of the original population.

For heterogeneous systems, the initial primary sample is the most difficult to obtain. Use of a sampling thief, sometimes called a grain probe or sampling spear, is needed. The advantage of a sampling thief is that much more of the powder bed is accessible because the sampling thief can sample from different points in the powder bed, thus helping to reduce sampling bias. Many types of sampling thieves are available, including: (1) the concentric sleeve with slotted compartments, (2) the concentric sleeve with grooves, sometimes called the open-handled probe, (3) the end sampler, and (4) the core sampler. Each type has its own unique operating procedures, as described below.

The concentric sleeve with slotted compartments is probably the most popular type of sampling thief used in the pharmaceutical industry. This type consists of two concentric tubes or cylinders in which the inner tube is divided into compartments. This design makes it possible to detect differences in the attribute of interest across the depth of the container. To collect a sample, the operator closes the compartments and inserts the sampling thief into the powder bed with the collection

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zone openings facing upward. The handle is turned to open the sample zones, then the handle is moved up and down with two quick short strokes to help fill the compartments. The sampling thief is then closed and removed from the powder bed. The operator should visually inspect the powder bed through its depth before emptying the sampling thief. The powder from the individual compartments can be combined on a clean surface or in a collection container. In certain situations the material from each compartment may be analyzed separately, that is, without mixing.

In the concentric sleeve with grooves (open-handled probe), the inner tube is not divided into compartments. The probe is first inserted into the powder bed with the groove open, the outer sleeve is rotated to close, and the sampling thief is then withdrawn from the powder bed. The probe's contents are emptied from the handle end by holding the probe upright and letting the sample slide out from the handle, a method more convenient than the one using the thief with slotted compartments. However, this type of thief makes it more difficult to perform visual inspection to examine for material inconsistencies according to depth.

An end sampler probe, often used to sample slurries, has a single entry zone at the bottom of the sampling thief. Frequently the end sampling zone is larger than the rest of the sampling thief. This feature is a disadvantage because the larger the probe, the more it perturbs the powder bed, possibly resulting in the introduction of sampling bias.

Core samplers have a hollow outer cylinder with a tapered outer wall on the open end. This probe is inserted into the powder bed, and the intrinsic cohesion of the particles keeps them from flowing out when the probe is withdrawn. The contents of the cylinder are then emptied into a clear container.

GENERAL CONSIDERATIONS

The most reliable and reproducible results in powder size measurements are obtained when the particle size ranges from 2 to 10 µm; otherwise, the powder is too cohesive and does not flow properly into the sampling thief. In addition, particles larger than about one-third the width of the slot give poor results. Samples should be taken from several sites throughout the container. The probe should be long enough to penetrate at least three-quarters of the depth of the powder bed, ensuring that material from all depths can be captured in the sample. The choice of sites should be dictated by an understanding (often subjective) of the powder bed's degree of heterogeneity, which may have been caused by handling or movement during transport. Sampling plans can call for the insertion of the probe either at random locations and random angles or at predetermined locations and angles. For example, the plan may call for the probe to be inserted at the center and at two locations near the edges. Also, many operators recommend that probes always be inserted at a 10° angle from vertical, which increases the range of locations sampled.

Some of the disadvantages of sampling thieves include the labor-intensive nature of the procedure. The probe must be physically inserted into the powder bed, often multiple times; the contents of the probe must be emptied; and then the probe must be thoroughly cleaned. For settled powder beds, the sampling probe can be difficult to insert. In addition, the sampling probe can introduce errors as a result of the following: fine particles can lodge between the inner and outer tubes; particles can fracture; fine particles can compact and not flow well into the sampling compartments; segregation can occur during flow into the sampling zone; and the act of inserting the probe can disrupt the powder bed by dragging powder from the top layers of the bed down through the bed.

Representative Lot Sampling

Statistically-based sampling plans are based on statistical principles and depend on the population's spatial heterogeneity and intrinsic variability. Statistically-based plans are efficient and allow the collection of a sufficient number of samples to yield the desired degree of certainty without collecting too many or too few samples for the test method, scale, product variation, risk requirements, and tolerance for a stated product's quality level or specification. The commonly used $\sqrt{N} + 1$ sampling plan given in *Table 1* is not a statistically based sampling plan and may result in collection of too few samples for small populations and too many samples for large populations. The use of statistically-based sampling plans is advantageous because it facilitates risk management. However, in cases where prior knowledge of the population to be sampled is insufficient, a nonstatistical sampling plan such as that given in *Table 1* can be considered.

Figure 4 illustrates the sample size selection scheme paths. The first choice is whether to use a statistical or nonstatistical sampling plan. Statistical plans are preferred when a variable attribute like particle size or drug content is being determined. General sampling approaches are outlined in *USP* general information chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩. Statistically-based lot acceptance sampling plans require a valid rationale with known quality levels for the determination of product lot characteristics. As noted, the application of statistical sampling plans, including lot acceptance sampling plans, requires specific and thorough knowledge of the material being sampled. Reference statistical sampling plans state the rationale for sampling as part of the sampling scheme. Manufacturers who use a statistically-based lot acceptance sampling method should refer to an appropriate standard such as ANSI/ASQ Z1.9-2003 for bulk materials or ANSI/ASQ Z1.4-2003 for multiunit or discrete populations. These standards are readily available via sources such as the American Society for Quality (http://www.asq.org/) or the American National Standards Institute (http://www.ansi.org/).

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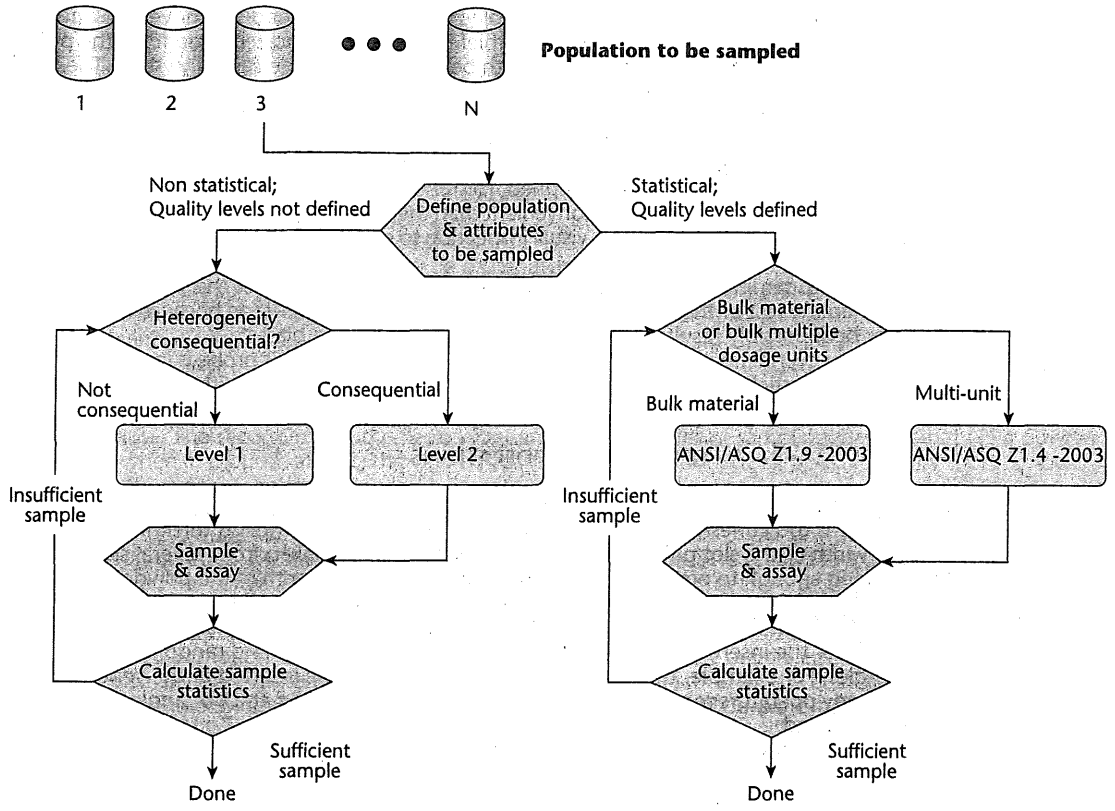

Figure 4. Sample size selection scheme.

If one is developing a nonstatistical sampling plan for which the quality level is not known, *Table 1* gives suggested sample sizes for the number of containers in the lot that should be sampled.

The *Level 1* sampling plan is relevant to materials when heterogeneity does not affect the analysis and the customer seeks to sample more than one container, when the sampling plan can be proportional to the square root of the number of containers received, and when the material comes from a known and trusted source. In such cases, the sample can be withdrawn from any point in the container. For adequately homogenous systems, scoop sampling from the top of the container is suitable.

The *Level 2* sampling plan involves a 50% increase in sample size when compared with *Level 1* and is used when a larger proportion of the number of containers is needed, for example, when a material's heterogeneity is suspected of being consequential and acceptance sampling quality levels are not defined, or when the material comes from a less trusted source. Depending on the material's degree of heterogeneity, a sampling thief may be used. However, if the degree of heterogeneity will not significantly affect the results for the attribute being sampled, then scoop sampling from the top of the drum may still be suitable.

Table 1 shows the number of containers, n, to be sampled for a lot segregated into N containers. Note that the value of n from the formula is rounded at 0.5 up to the next higher integer. For example, if N = 6: for *Level 1*, $n = \sqrt{6} + 1 = 3.45$ rounds to n = 3; for *Level 2*, $n = 1.5 \times \sqrt{6} = 3.67$, which rounds to n = 4.

Table 1

N (Number of Containers Comprising Lot)	n (Sample Size)	
	Level 1	Level 2
$N \leq 3$	All	All
$N \geq 4$	$\sqrt{N} + 1$	$1.5 \times \sqrt{N}$

These initial decisions, as illustrated in *Figure 4*, are often difficult and sometimes must be made without sufficient information. If there is uncertainty about which method or level is appropriate, sometimes a quick, small-scale informal test of the system may help determine the best way to proceed. In addition, for some systems and attributes, the *Level 1* and *Level 2* sampling plans may result in oversampling. For example, when one is sampling for identification from the same lot, the suggested levels may result in collecting more samples than are statistically needed; in such cases, the statistically-based sampling plans referenced in *Figure 4* can be used.

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Sample Collection

Acquiring a representative sample from a lot of bulk powder is a difficult procedure that requires special consideration, and the basic procedures for acquiring a representative sample are discussed below. Note that every situation requires techniques that are appropriate for the given population to be sampled. The methods presented here are applicable to the sampling of static powders stored in midsize bulk containers such as 1-ton super sacks, 50-kg drums, or 50-lb bags. These methods are not necessarily applicable to the sampling of liquids, large storage containers such as train cars or silos, or in-process systems such as blenders or moving conveyer belts. In addition, the procedures described here are most applicable to particles in the size range from approximately ~1 µm to approximately ~1000 µm. Significantly smaller or larger particles require special procedures that are not covered here.

PRIMARY SAMPLE COLLECTION

Lot acceptance samples are generally transferred or delivered in containers. To collect a representative primary or gross sample (see *Figure 1*), the appropriate container or containers must first be selected from the population of N containers; second, a representative sample must be withdrawn from each of the selected containers.

Container Selection

To avoid bias and other sampling errors, the containers to be sampled must be randomly selected. To make a random selection, first number all containers in the lot, then use a random number table (or computer-generated random numbers) to choose from which container or containers to withdraw the samples.

For systems in which containers are grouped together in such a manner that many of the individual containers are not practically accessible (e.g., 50-lb bags stacked and bound in shrink wrap on a pallet), the sampling plan may need to take into account the larger container, in addition to the smaller container, as a sampling unit, in order to ensure a representative sample.

Withdrawing Sample from a Container

CONTAINER TYPES

The three most popular container types are the bag, drum, and super sack. Because bags are generally closed and not resealable, special sampling thieves, sometimes called bag triers, have been designed to puncture the bag. If the system to be sampled is heterogeneous, the samples should be obtained from the bottom, center, and top of the bag; and, depending on how the bags are stacked on the pallet, they should also be sampled from the front and the back. When sampling from bags, particular attention should be paid to the corners, because they can disproportionately trap fine particles. If no bag trier is available, use a knife to cut open the bag for sampling. When sampling from a bag, be sure to clean the external surface sufficiently that the sample is not contaminated and foreign material is not introduced into the bulk material. Once the sample has been taken, place a compatible material over the hole in the bag, then fix this patch with an appropriate adhesive tape. Depending on the heterogeneity of the drum, a scoop or a sampling thief is used. Super sacks are large sack containers that usually have a fill spout on the top and a discharge spout on the bottom. For adequately homogeneous material, scoop sampling is appropriate; but if there is any concern about the heterogeneity of the material, a thief should be used. The large size of super sacks makes the use of a thief more important for representative sampling than in the case of a drum or bag, in order to limit potential delimitation error.

Sample Handling

The samples collected can be either assayed individually or combined; then a subset of the gross sample can be assayed, as depicted in *Figure 1* and described below. Sample increments should be combined on a clean, dry surface or in a suitable container or bag. All containers with which the sample comes into contact should be inert and should not chemically or physically react with the sample. In addition, samples should be accurately labeled and good records kept. A portion should be kept for possible future analysis.

PRIMARY SAMPLE SIZE REDUCTION

As mentioned above, the primary sample typically consists of multiple samples taken from containers and mixed together. To obtain an analysis or measurement sample (*Figure 1*), the gross or primary sample must be reduced to a size appropriate for the analytical method. Gross or primary sample size reduction is an often overlooked aspect of a sampling plan, but it is an important step. The factors that cause segregation in a container can also cause segregation in the primary sample, and any bias in the size reduction method for the primary sample will lead to erroneous results. The advantage of secondary samples is that the mass has been reduced to a point at which it is much easer to obtain a representative sample because every element in the powder bed is readily accessible. Such accessibility makes it easier to adhere to sampling best practices. Generally speaking, sample measurement takes place under either wet or dry conditions; the choice is dictated by the requirements of the analytical method. For example, the Coulter counter requires that samples be uniformly suspended in an electrolyte, but other methods, like sieving, are typically performed with dry powders.

Before dividing an agglomerated sample, the agglomerates should be broken apart by a suitable technique such as sieving.

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Dry Analysis Methods

Many laboratory devices are available for the reduction of the primary sample to an analytical sample. The three most important methods used in the pharmaceutical industry are: (1) scoop sampling, (2) cone and quartering, and (3) the spinning riffler or rotary sample divider (manual method of fractional shoveling); see *Figure 5*.

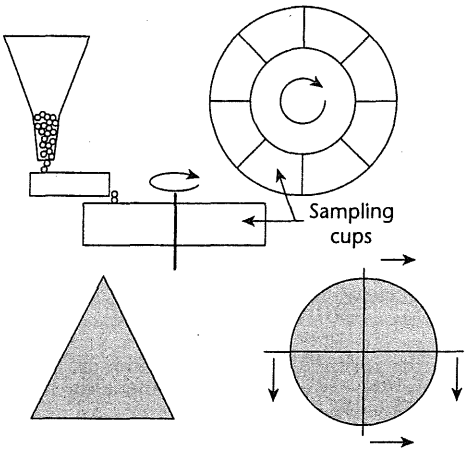

Figure 5. Two procedures for dividing samples. Top: spinning riffler, in which a circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Bottom: cone and quartering. (Cone, left, is flattened and quartered; quarters can be formed into cones and further subdivided.)

SCOOP SAMPLING

Scoop sampling is done as previously described, but generally with a smaller scoop or spatula. Great care must be taken when removing material from the primary sample, because this material could be highly segregated as a result of handling. Scoop sampling is appropriate for homogeneous or cohesive powders. However, if the powder is prone to segregation, scoop sampling can introduce significant errors. Moreover, scoop sampling has several serious disadvantages. First, the method depends on the operator's deciding from which part of the primary sample to scoop the material and what quantity of the sample to extract, which are features that can introduce operator bias. Second, in scoop sampling, operators have a natural tendency to withdraw the sample from the free surface, which is highly prone to segregation and is not representative of the bulk. Third, operators need to avoid creating a heap where rolling segregation can occur, because material could fall off the edges of the spatula or scoop and bias the sample. Ideally, the operator should make some attempt to mix the primary sample before using the scoop, but this too can exacerbate segregation problems and should be done only with great caution.

CONE AND QUARTERING

Cone and quartering is done by pouring the primary sample into a symmetric cone on a flat surface. The cone is then flattened by a flat surface such as a spatula, and is divided into four identical quarters (*Figure 5*). One quarter is taken as the sample. This procedure can be repeated (e.g., quarter-samples can be subdivided into quarters) until the desired sample size is obtained. The theory of this method is that when a symmetric cone is created, all the segregation processes also occur symmetrically around the cone, and hence symmetry is used to mitigate the effect of segregation. In practice, it is very difficult to actually make a symmetric powder cone, and the method becomes very operator-dependent and often unreliable. Differences in how operators form the heap and subdivide it can lead to a lack of precision and significant errors. In addition, if the method is done more than once, errors can propagate each time the cone and quartering is performed. Some experts do not recommend this method.

SPINNING RIFFLER

A spinning riffler (*Figure 5*) includes a series of containers mounted on a circular holder. The circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Once the material has been divided among the different holders, an individual holder can be removed for testing or further sample division. The angular velocity of the circular holders and the amplitude of the vibratory feeder can be controlled to accommodate powders with different flow properties. The holder velocity and feed rate should be adjusted so that the containers fill uniformly and so that a heap does not form on the vibratory feeder. Spinning rifflers are available in different sizes, making possible subdivisions of powders ranging from a few milligrams to hundreds of grams. The only drawbacks of the spinning riffler are the time required to process the sample and clean the device, and the capital expense. Despite these minor disadvantages, the spinning riffler is by far the best method for subdivision of free-flowing powders.

Fractional shoveling is the manual version of the spinning riffler. In this method, scoop samples are taken from the original sample and placed into a sufficient number of aliquots, and then subsequent scoops are taken from the original sample and placed into one of the aliquots in sequential order. This process is repeated until the original samples are gone. Then one of

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the aliquots is randomly taken as the reduced sample. As is the case with all manual methods, operator error and variability can be significant factors.

Wet Analysis Methods

Wet analysis methods require dispersing the sample in a liquid suitable for analysis, and then withdrawing an aliquot using a syringe or pipet. Effective secondary sampling requires making a stable homogenous suspension (i.e., the sample must be stable from the time of formation of a suspension to the time when the analysis is complete). Some important factors in wet analysis are sample solubility in the dispersion vehicle, aggregation of sample, the use of suspending agents, and deaggregation of primary particles in the dispersion vehicle. Even though a uniform suspension is created, the sample should be homogenized, typically by shaking, immediately before withdrawing the sample with a syringe or pipet. The diameter of the syringe or pipet should be large enough so that particles are not excluded and clogging does not occur. The diameters of the largest particles should not exceed 40% of the syringe or pipet tip diameter. If for practical reasons the amount of material from the primary sample is too large, the sample size should be reduced before a suspension is made. To reduce the sample size, use the methods described above in the *Dry Analysis Methods* section. As a precaution, collect and retain enough sample to repeat all tests a minimum of five times.

APPENDICES

Appendix 1: Subsampling Examples

The examples provided below describe the importance of material particle characterization during the selection of an appropriate sample mass. Four examples are presented. In the first example, similarity in the fundamental or intrinsic material characteristics is assumed. In the second example, the density of the heavy metal analyte being measured is changed. In the third example, the effect of changing the particle size is evaluated. In the fourth example, the adequacy of the fundamental particle characteristics in a formulation needed for a given unit dose or mass is evaluated.

EXAMPLE 1. SAMPLE MASS DETERMINATION

Assuming the lot size is 1 kg, the maximum particle diameter is 1000 µm, and the concentration of the analyte is expected to be 1%, what sample mass of round, equal-sized and -shaped 1000-µm particles with a density of 1 g/cm³ would be needed to estimate the average concentration of the analyte with a percent relative standard deviation (%RSD) of 5%?

Rearranging equation 3, one can estimate the sample mass as shown in equation 5:

$$m_{sample} \approx \frac{1}{\frac{S_{fse}^2}{f_{shape}\, g_{CF}\, C_{max}\, ld_{max}^3} + \frac{1}{m_{lot}}} \qquad (5)$$

The compositional maximum heterogeneity (c_{max}) can be estimated by considering the analyte and matrix density (λ_a and λ_m, respectively, and their average λ) and analyte concentration (a_L) (equation 6):

$$C_{max} = \frac{(1-a_L)^2 \lambda_a \lambda_m}{a_L \bar{\lambda}} \qquad (6)$$

For low analyte concentrations, the compositional maximum heterogeneity is simplified to equation 7:

$$C_{max} \approx \lambda_a / a_L \qquad (7)$$

For high analyte concentrations, the compositional maximum heterogeneity is simplified to equation 8:

$$c_{max} \approx \lambda_m (1-a_L) \qquad (8)$$

The shape factor is approximated by equation 9:

$$f_{shape} \approx \text{Volume} / d^3 \qquad (9)$$

Where d is the nominal particle diameter for a sphere, and the shape factor is [(4/3)π/8], or approximately 0.5.

The granulometric factor can be approximated by the typical minimum diameter noted as the 5th percentile size, divided by the typical maximum diameter noted as the 95th percentile size, as shown in equation 10:

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$$g_{CF} \approx d_{5\%}/d_{95\%} \qquad (10)$$

Because all particles are the same size, the granulometric factor, g_{CF}, is 1.0. Because the analyte exists in a state liberated from the matrix particles, the liberation factor is also 1.0. The sample mass for a 5% RSD (using equation 5) is then:

$$m_{sample} = \frac{1}{\frac{0.05^2}{0.5 \times 1 \times 100 \times 0.1^3} + \frac{1}{1000}} = 19.6 \text{ g}$$

A sample mass of 19.6 g will provide a sampling error of approximately 5% RSD. Note that in this example the particle characteristics are simplified to demonstrate that a lot mass of 1000 g contains 2×10^6 particles of 0.5 mg mass. The sample mass of 19.6 g contains approximately 39,216 particles, yielding a 5% RSD, using the binomial distribution where p is the concentration of the analyte (a_L) and n is the number of particles sampled, as shown in equation 11.

$$\text{Binomial RSD} = \sqrt{(1-p)/np} = \sqrt{(1-0.01)/39{,}216 \times 0.01} = 0.0498 \approx 0.05 \qquad (11)$$

(See *Table 2* for a summary of calculations.)

Table 2. Summary of Calculations for *Example 1*, Equal-Sized and -Shaped Particles

m_{Lot} (g)	d (cm)	f_{shape}	g_{CF}	c_{max}	a_L	λ_a(g/cm³)	l	m_s (g)
1000	0.1	0.5	1	100.0	0.01	1	1	19.6
Mass per Particle P_s $d^3 f_{shape} \lambda_a$			Particles in 19.6 g $m_s/(P_s/g_{CF})$				Binomial RSD $p = a_{(L)} = 0.01$ $n = 39{,}216$ (Eq. 11)	
0.005			39,216				0.05	

In determining the required sample mass, it is assumed that the sample is representative of the population. Moreover, when using a single representative sample, it is assumed that the uniformity of the sample mass is consistent with the remaining population. Note that the granulometric and liberation factors allow proportional adjustment of the sample size, depending on the nature of the particles. The inclusion of a liberation factor in the equation allows for particles to exist with a proportion of the analyte residing within every particle or a proportion thereof. The granulometric factor permits adjustment of the sample mass by accounting for the relationship in size between the smallest and largest particles represented in the lot.

This approximation also can be applied to liquid suspensions in which each particle is considered discrete and the sample can be characterized with respect to size, density, mass, and volume.

EXAMPLE 2. HEAVY METAL

In this example, it is assumed that the analyte is the heavy metal lead, with a density of 11.34 g/cm³, with a limit of not more than 5 ppm, where the shapes of the particles are cubes ($f_{shape} = 1.0$), the particles are approximately 50 µm, and a 5-g sample is taken from screened material ($g_{CF} = 0.55$). On the basis of equation 3, the %RSD is 17.7%. Using equation 5, one finds that a sample mass of approximately 60 g is needed to achieve a 5% RSD, assuming that a_L is equal to the limit allowed and that the analyte cannot be assumed to be liberated from the material (l = 1.0). (See *Table 3* for a summary of calculations.)

Table 3. Summary of Calculations for *Example 2*, Heavy Metal

m_{Lot} (g)	d (cm)	f_{shape}	g_{CF}	c_{max}	a_L	λ_a (g/cm³)	l	m_s (g)
1000	0.005	1.0	0.55	2.3×10^6	5×10^{-6}	11.34	1.0	58.71

If the sample were assumed to be homogeneous (l = 0.1) with respect to presence of the analyte with all particles, then a sample mass of 6.2 g would be required. Moreover, if the shape of the particles were between round and cubic ($f_{shape} = 0.8$), then a sample mass of 5 g would be required to complete the analysis.

EXAMPLE 3. SUBSAMPLING

Ideal sampling, as noted earlier, is fundamental to understanding the important role of subsampling. In many instances it is desirable to reduce the sample size in a manner that results in a representative sample and lessens the need to test a large sample mass. In some cases the particle size and compositional heterogeneity can result in an unwieldy sample mass. This may occur with larger-sized particles or when a composite sample of many containers is required. Samples with larger-sized particles may need to be physically reduced.

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For example, using *Example 2* above, if the maximum particle size were 1000 µm or 1 mm, then a 997-g sample would be suggested by equation 3. Reducing the particle size by grinding and subsampling to achieve a predetermined sampling %RSD may require subsampling more than once to achieve the desired particle size. For example, the entire sample may be reduced to 100 µm to reduce the %RSD to approximately 3%; then, with ideal sampling, a subsample could be selected and entirely reduced to 50 µm to achieve a 5% RSD. Finally, a 5-g subsample could be correctly taken and tested. If certain particles have a large size with high concentration of the analyte, then samples should be selected to ensure that at least 1, but preferably at least 5–6, particles would be selected with 95% probability or chance of selection.

EXAMPLE 4. MINIMUM UNIT DOSAGE MASS

A formulator would like to know the minimum mass required for a dosage form to ensure with 95% confidence a unit dosage of 1% active drug powder. The drug and the excipient have a similar round shape (f_{shape} = 0.5) and a density of 0.33 g/cm³. The active drug is milled to 1 µm, but the size of excipients can be as large as 200 µm. The value for g_{CF} is taken from equation 10 using the expected range of the excipient that accounts for 95% of the formulation, as 10 µm/200 µm, or g_{CF} = 0.05. The quantity c_{max} from equation 7 is taken as 0.33/0.01. The drug particles are completely liberated from the excipient. The batch size is 100 kg.

A minimum sample mass of approximately 3 mg is needed to ensure with 95% confidence (2 RSDs) that the average drug content is 0.9%–1.1%. The proposed dosage form has an active concentration of 100 µm/10 mg total unit mass. The unit dosage form mass is adequate, but the formulation requires that the mixing process, unit dosage production, bulk sampling device, and lab sample preparation or subsampling from bulk samples result in equal probability of selection of drug particles. Only if these conditions for mixing, production, sampling, and testing are met can it be reliably demonstrated that the unit dosage and test determination acceptance criteria of 1% (0.01 µg/mg) are met. Acceptable outcomes of such testing also indicate that the particle size, shape, and density must be controlled. For example, an increase in the sizes of particles to 500 µm results in a need for a 42-mg sample mass and dose. Assuming a cubic, as opposed to a rounded, particle increases the sample mass to 5 mg, which for a fixed dosage form mass may result in less room for the variation contributed by other characteristics, or in lesser confidence. If the acceptance criteria were changed to 0.95%–1.05%, requiring a 1% RSD, then the minimum sample mass would increase to approximately 70 mg. (See *Table 4* for a summary of calculations.)

Table 4. Summary of Calculations for *Example 4*, Minimum Unit Dose Mass

m_{Lot} (g)	d (cm)	f_{shape}	g_{CF}	c_{max}	a_L	λ_a (g/cm³)	l	m_s (g)
10^5	0.02	0.5	0.05	33	0.01	0.33	1.0	0.00264

Appendix 2: Material Characterization and Sampling

Specific and thorough knowledge of the material's synthesis, composition, and usage is critical to developing a bulk material sampling plan. Material characterization is important because bulk material can exist in many forms throughout the material process flow. As illustrated in *Figure 6*, the type of sampling can vary by process step and ultimately affects the use of the material in the drug product. Appropriate material characterization considers the material process step, the type of sampling, the objective of the process step, and ultimately the drug product.

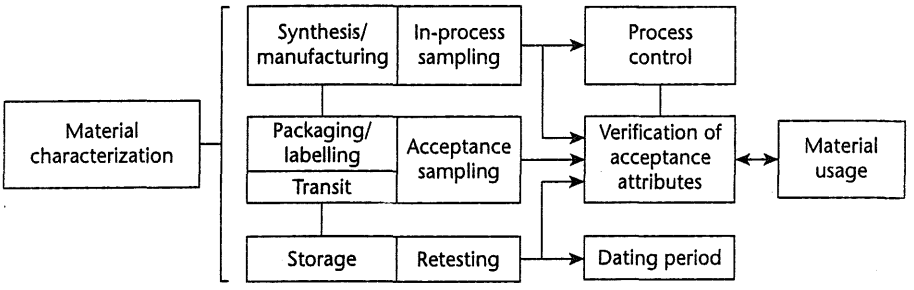

Figure 6. Material process flow.

For example, material can be synthesized or mixed in a large container where sampling may be limited or ideal for the characteristic needed. If the characteristic is important but the sampling conditions are not ideal, perhaps because of the heterogeneity of a powder mixture, then sampling for that characteristic may be more appropriately performed at a different stage upstream or downstream to assess the heterogeneity of the contents and ensure ideal sampling. This is sometimes performed to reduce the sampling dimension. The sampling dimension is reduced when the 3 dimensional bulk container space is sampled in a 1 or 2 dimensional stream sampled over time. Reducing the spatial sampling dimension may result in conditions that will allow for more accurate measurement of the heterogeneity of material while limiting sampling error through ideal sampling.

Acceptance attributes (see *Table 5*) depend on material characterization and process. Acceptance attributes may be applicable throughout the life of the bulk material. Both the number and size of samples require an understanding of the material's variation.

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Table 5. Examples of Acceptance Attributes

Acceptance Attributes			
Physical	**Chemical**	**Microbiological**	**Packaging**
Particle size Viscosity Density	Purity pH Identity Strength	Sterility Pyrogens Microbial load	Label accuracy Integrity

Appendix 3: Additional Sources of Information

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Add the following:

▲⟨1099⟩ LIMIT ON NUMBER OF LARGE DEVIATIONS WHEN ASSESSING CONTENT UNIFORMITY IN LARGE SAMPLES

INTRODUCTION

The uncertainty around the application of the zero tolerance criterion (ZTC) to sample sizes larger than 30 may inhibit the collection of uniformity data from large samples. This chapter provides a process for limiting the number of observed results that fall outside of the ZTC (c_2), as described in *Uniformity of Dosage Units* ⟨905⟩, when samples larger than 30 are collected. It should be noted that the criterion described in this chapter is not intended as a batch release test, nor as a replacement of or alternative to ⟨905⟩. It also is not intended as an extension to go beyond second tier testing in ⟨905⟩. The use is solely to help judging if a large data set is consistent with the ZTC element of ⟨905⟩; whether the large data set complies with the complete set of requirements in ⟨905⟩ must be decided by other means.

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The ZTC in ⟨905⟩ states that no individual content of any dosage unit can be less than [1 – (0.01)(*L2*)]*M* nor more than [1 + (0.01)(*L2*)]*M*, where *L2* is 25.0% unless otherwise specified in the applicable monograph, and *M*, the "reference value", depends on the sample mean, $\bar{X}$ (expressed as a percentage of the label claim), as follows:

$M = 98.5\%$ if $\bar{X} < 98.5$, $M = 101.5\%$ if $\bar{X} > 101.5$, and $M = \bar{X}$ otherwise, with a sample size (N) of 30

PROCEDURE

When a sample that includes the contents of more than 30 units has been collected, the following procedure can be used to confirm that the results in that sample are consistent with the ZTC of ⟨905⟩. The criterion is applicable both when the content is determined directly by assaying a number of units and when the content is determined indirectly by weighing the units in the situations allowing this as described in ⟨905⟩. The procedure is as follows:

1. Express individual results $x_1, x_2, \ldots, x_N$ as a percentage of the label claim
2. Calculate the mean ($\bar{X}$) of the contents of the N units in the sample
3. Calculate the reference value M: $M = 98.5\%$ if $\bar{X} < 98.5$, $M = 101.5\%$ if $\bar{X} > 101.5$, and $M = \bar{X}$ otherwise
4. Determine S_N, the number of sample results less than [1 – (0.01)(*L2*)]*M* or more than [1 + (0.01)(*L2*)]*M*, where *L2* = 25.0% unless otherwise specified in the applicable monograph
5. The sample is consistent with the ZTC of ⟨905⟩ if $S_N \le c_2$, where c_2 depends on the sample size as detailed in *Table 1*

Table 1. Limit on Number of Observed Results Falling Outside of the ZTC Based on Sample Size

N	c_2
31–100	0
101–181	1
182–265	2
266–353	3
354–442	4
443–533	5
534–624	6
625–717	7
718–810	8
811–903	9
904–998	10
999–1092	11
1093–1187	12
1188–1283	13
1284–1379	14
1380–1475	15
1476–1571	16
1572–1667	17
1668–1764	18
1765–1861	19

Values of c_2 for other sample sizes (N) are determined as

$$c_2 = \max\left\{c: \sum_{i=0}^{c} \binom{N}{i} f^i (1-f)^{N-i} \le 0.75\right\},$$

where $f = 1 - 0.75^{1/30} = 0.00954357$. The c_2 value can be calculated in a spreadsheet with a cumulative binomial function. ▲ (Postponed on 1-Mar-2019)

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⟨1102⟩ IMMUNOLOGICAL TEST METHODS—GENERAL CONSIDERATIONS

INTRODUCTION

This general information chapter provides a high-level description of principles for immunological test methods (ITMs) that can be used in specified monograph tests, along with information and approaches to analytical development and validation for ITMs. The scope of this chapter is to provide general information that is applicable to all ITMs. The chapter provides a foundation for specific chapters about different types of ITMs, e.g., *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩ (proposed), and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩. This suite of general information chapters is related to the bioassay general information chapters. Use of ITMs for process monitoring, diagnosis, and evaluation of clinical response, assessment of pharmacokinetics/pharmacodynamics/absorption, distribution, metabolism, and excretion (PK/PD/ADME), and other product characterization (nonrelease testing) is outside the scope of this chapter.

The basis of all ITMs used to measure a quality attribute of a biologic drug substance or drug product is the highly specific noncovalent binding interaction between an antibody and antigen. The antigen typically is an analyte of interest (e.g., protein, carbohydrate, virus, or cell), and the binder is usually an antibody (e.g., monoclonal antibody or polyclonal antiserum). ITMs are applicable to molecules that are either directly antigenic (immunogens) or can be rendered indirectly antigenic (haptens). The measurand in ITM is directly related to a quality attribute of the product under test.

ITMs are valuable because they exhibit high sensitivity and specificity for an analyte in complex matrices. They typically are used for qualitative and quantitative assessment of both an antibody and antigen, but their application also extends to the measurement of hapten, complement, antigen–antibody complexes, and other protein–protein interactions. These properties of ITMs allow their use for assessing identity, potency (strength), purity, impurities, stability, and other quality attributes of biological drug substances and drug products.

ITMs are useful for many applications because they can measure molecules over a wide range of sizes and binding types. In general, antibodies are stable during various chemical modifications that do not have a significant adverse influence on interactions with an antigen. Antibody molecules tend to withstand moderate acidic and alkaline pH changes better than other proteins do. Because of this characteristic, a variety of ITMs with high degrees of sensitivity and specificity are possible. The ability to accelerate contact between an antigen and antibody enables ITM formats that provide rapid or real-time results.

Generally, ITMs have higher precision and shorter turn-around time than do traditional biologically-based (i.e., cell-based and animal) assays. Although in some cases these advantages can support the replacement of a biological assay with an immunoassay, such changes should be approached systematically and with caution. Often it is challenging to prove the equivalence, or comparability, of results from bioassays and immunoassays because the interaction between antigen and antibody may not reflect the functional attributes observed in bioassays.

One major limitation of ITMs compared to physicochemical methods (such as liquid or gas chromatography) is that the latter generally are more precise and can simultaneously identify a set of impurities or unexpected substance(s). Another major limitation is that generally ITMs operate at high molar dilutions at which they are sensitive to disturbances caused by environmental factors in the sample matrix (i.e., matrix effects). Matrix effects can depend on ITM format and are not fully understood. Their specificity, a hallmark of ITMs, is sometimes compromised by structural or sequence similarities between the analyte and a closely related molecular impurity (cross-reactivity).

Most ITMs reflect physical interaction (binding) between an antigen and antibody and not the analyte's functional properties. Therefore, analysts must pay attention in the selection and execution of ITM format. Cell-based ITMs that can provide functional information about the analyte are beyond the scope of this chapter.

GENERAL CHARACTERISTICS OF ITMS

ITMs are based on the principle of specific, noncovalent, and reversible interactions between an antigen and antibody. In general, the primary antigen–antibody reaction is brought about by complementarity, which creates macromolecular specificity. This noncovalent interaction determines the degree of intrinsic affinity. Intrinsic affinity contributes to functional and/or relative affinity that depends on factors like reaction phase and valency, which in turn determines the degree of reversibility of an interaction. A better understanding of factors that affect antigen–antibody interactions provides the rationale for the development of a suitable ITM format (e.g., solid or liquid phase, competitive or noncompetitive binding, etc.).

A defining characteristic of ITMs is that they employ an antigen (or hapten) and antibody. In addition, ITMs may contain companion molecules such as complement components. The components of ITMs are defined as follows:

- **Antigens**—Comprise a wide range of molecules that are capable of binding to the antibody in a specific interaction. Generally, part(s) of an antigen (the immunogenic epitope[s]) is/are capable of eliciting antibody response.
- **Haptens**—Small molecules that, by themselves, are not capable of eliciting an antibody response but are capable of eliciting an immune response when attached to a large carrier such as a protein. Antibodies produced to a hapten–carrier adduct also may bind to the small-molecule hapten in a specific interaction.
- **Complements**—Companion molecules that, under certain conditions, aid in the functionality of antigen–antibody complexes but are not required for antigen–antibody or hapten–antibody interaction.
- **Antibodies**—Proteins with regions that impart a high degree of specific binding to antigens (and haptens). The structural elements of an immunoglobulin G (IgG) antibody are shown in *Figure 1*.

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In addition to these components, ITMs require some means to detect or monitor the binding reaction between the antigen and antibody.

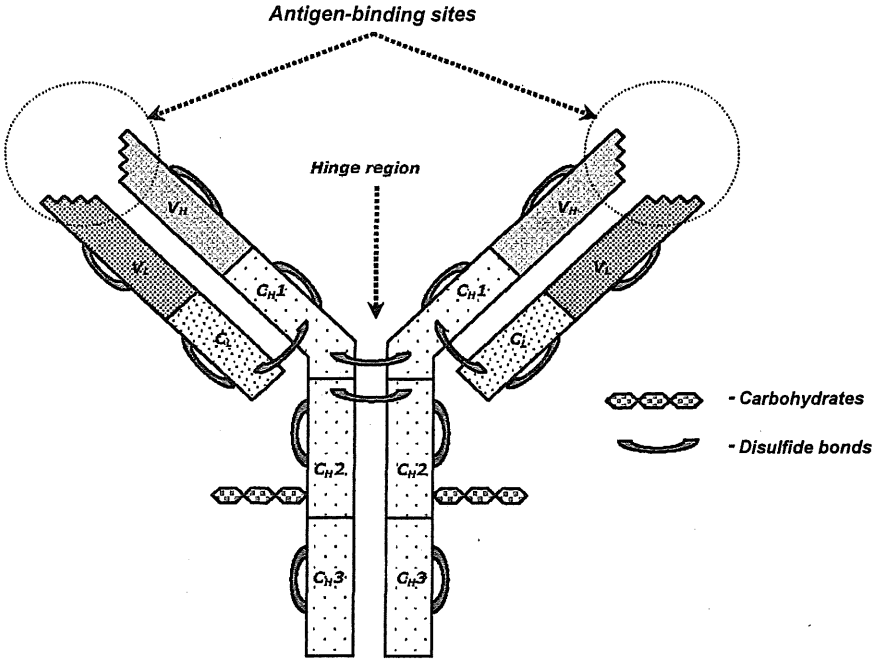

Figure 1. The structure of IgG. The IgG molecule is characterized by a distinctive domain structure of heavy (H) and light (L) chains, both of which are divided into variable and constant regions (V and C, respectively). Light chains consist of V_L and C_L domains, and heavy chains consist of a variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3). All domains are stabilized by disulfide bonds, and C_H2 domains contain carbohydrates. The flexible hinge region between the C_H1 and C_H2 domains allows the independent behavior of two antigen-binding sites formed by variable domains.

TYPES OF ITMS

Measurement of antigen–antibody binding can be performed in a variety of assay types and formats: solid or liquid phase, manual or automated, labeled or nonlabeled, competitive or noncompetitive, qualitative or quantitative, homogeneous or heterogeneous, or combinations of some of these. The distinguishing characteristic of all these assays is the binding of an antibody or antigen to the analyte (which can be an antigen or antibody as well), followed by detection of the antigen–antibody complex. Although many different formats can be used for the binding reaction, along with different methods for detection, quantification of the analyte in the test article is always performed by comparison of the measurement to a reference standard. Thus a number of ITM technologies support investigations of product quality. Commonly used assay designs include enzyme-linked immunosorbent assay (ELISA), Western blotting, flow cytometry, competitive enzyme-linked immunosorbent assay, surface plasmon resonance (SPR), rate nephelometry, radioimmunoassay (RIA), radial immunodiffusion, precipitation, and agglutination. These methods are described below.

Enzyme-Linked Immunosorbent Assay

An ELISA is a quantitative, solid-phase immunological method for the measurement of an analyte following binding to an immunosorbent and its subsequent detection using enzymatic hydrolysis of a reporter substrate either directly (analyte has enzymatic properties) or indirectly (e.g., horseradish peroxidase– or alkaline phosphatase–linked antibody subsequently bound to the immunosorbed analyte). The analyte usually is quantitated by interpolation against a standard curve of a reference material. General information chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩ discusses ELISA in greater detail, including ELISA development for quantitative analysis.

Western Blotting

A Western blot is a semiquantitative or qualitative method for measurement of a protein analyte that has been resolved by polyacrylamide gel electrophoresis and subsequently transferred to a solid membrane (e.g., nitrocellulose, nylon, or polyvinylidene difluoride). Detection can be achieved directly by reacting with a labeled primary antibody (antibody specific to the analyte of interest) or indirectly by reacting labeled secondary antibody (antibody against the primary antibody) to the primary antibody bound to the membrane-immobilized antigen. The label can be a radioisotope or an enzyme that uses the

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substrate to produce color, fluorescence, or luminescence. This method is semiquantitative, especially when proteins are present in low concentration and in very complex mixtures. It is commonly used in early process development (e.g., antibody screening, protein expression, protein purification, etc.). Western blotting is a powerful method for analyzing and identifying proteins in complex mixtures, particularly after separation using 2-dimensional gel electrophoresis, which separates proteins based on size and charge (pI).

Flow Cytometry

Flow cytometry is a laser-based semiquantitative technology that permits measurement of fluorophore-conjugated probes as they interact with their respective ligands on cells or particles. More details for flow cytometry can be found in *Flow Cytometry* ⟨1027⟩.

Surface Plasmon Resonance

SPR is a quantitative method for measurement of an analyte in a sample where the antibody–antigen complex formation can be measured in real time at the interface of a liquid and solid (e.g., gold surfaces or particles). The measurement taken is the real-time change in refraction of a polarized light and occurs during the formation of the antibody–antigen complex, resulting in changes to the plasmon resonance minima (i.e., the sensorgram). The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay. More details for SPR can be found in general information chapter *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩.

Rate Nephelometry

Rate nephelometry is a quantitative method for measurement of an analyte in a sample in solution by measuring the light scatter introduced by small aggregates formed by the antigen–antibody complex. The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay.

Radioimmunoassay

RIA, a sensitive ITM first developed in the 1950s, is a quantitative method for measurement of an analyte in a sample. RIA usually uses a competitive antibody–antigen binding reaction, but it also can be used in sandwich immunoassay format, including immunoprecipitation. In competitive RIAs the analyte competes for binding with a radiolabeled (e.g., using ^{125}I or ^{3}H) reference antigen that is identical to the analyte; therefore, the analyte and the antigen both compete for binding to a fixed and limiting dilution of a specific (often polyclonal) antibody. The radiolabeled antigen is present in excess. The same unlabeled antigen in the test sample competes in binding to the same site on the antibody, which is present in a fixed quantity. Binding of the unlabeled antigen to the antibody leads to the displacement of the labeled antigen, resulting in a decrease in the radioactivity of the antigen–antibody complex fraction. To separate the antigen–antibody complex from the excess unbound antigen, the complex generally is either precipitated with a secondary antibody (or protein G) immobilized on a solid matrix (e.g., glass or resin beads) or with an already immobilized primary antibody. The quantity of analyte usually is determined by interpolation against a standard curve of a reference material, where a fixed amount of antibody and radiolabeled antigen is mixed with an increasing amount of unlabeled antigen. Hence, even a small quantity of unlabeled antigen will result in a relative quantitative decrease in total bound radioactivity.

Single Radial Immunodiffusion

Single radial immunodiffusion (SRID or SRD) is a quantitative method for measurement of an analyte in a sample by measuring the diameter of the ring of precipitin formed by the antigen–antibody complex. Antigen is applied to a well in a gel infused with a constant level of antibody. Solutions with higher concentrations of antigen diffuse farther before being saturated with antibody and then precipitated. The quantity of analyte is determined by comparison to a reference standard curve measured by the same assay.

Precipitation

The underlying principle for this method is that the interaction of a multivalent antibody and antigen leads to the formation of a complex. In some cases a visible precipitate is formed. Other immunoprecipitation techniques involve the use of Protein A or Protein G beads to capture the antigen–antibody complex and facilitate the separation of the antigen–antibody complexes from the other antigens in the solution. Precipitation is not commonly used for quantitative analytical purposes because of the time required (days to complete), lack of sensitivity, and requirement for large quantities of antigen and antibodies.

Agglutination

Agglutination and inhibition of agglutination, respectively, provide qualitative and quantitative measures of certain antigens and antibodies. Inhibition of agglutination is a modification of the agglutination reaction that provides higher sensitivity to detect small quantities of proteins, chemicals, viruses, and other analytes. The principle of agglutination is similar to that for precipitation except that the interaction takes place between an antibody and a particulate antigen and leads to a visible clump or agglutination. The most common example of this application is for blood typing (i.e., A, B, or O antigen).

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CHOICE OF ITM

When choosing an ITM, analysts should consider sensitivity and specificity as well as the complexity of the sample. *Table 1* provides an assay developer with a comparative view of the advantages and disadvantages of a variety of ITM formats. The intended application of the ITM should govern the choice of the most suitable format.

Table 1. ITMs Used in Biopharmaceutical Laboratories

Method	Advantages	Disadvantages	Typical Industry Uses
ELISA	• High sensitivity • Often wide dynamic range • High throughput • Low cost	• Multistage process highly dependent on proper execution of each stage • Wash steps add time and often biohazardous waste • Reagent labeling required	• Potency assessment • Specific protein concentration analysis in complex samples • Protein identification • Purity assessment • Immunogenicity assessment
Western blot	• Gives information about antigen size and/or charge • Allows separation of various antigens (or degradation/aggregation products) bearing same epitope • Can tolerate complex mixtures	• Typically works only with linear epitopes • Labor intensive • Low throughput, output • Subject to interpretation • Immobilization can alter binding • Limited to proteins	• Protein purity assessment • Protein stability assessment • Protein identity test
Flow cytometry	• High throughput • Highly automated	• Use limited to cells, particles, and samples bound to beads • Sensitive to aggregates and sample matrix	• Potency assessment • Cell identity in cell-therapy products
SPR	• Direct detection of binding • Can measure affinity precisely, including on and off rates	• Immobilization can alter binding • Regeneration can alter binding • Low throughput, output	• Immunogenicity assessment • Potency assessment • Specific protein concentration analysis in complex samples
Rate nephelometry	• Easily automated • Rapid	• Small detection range • High background for turbid samples	• Assay for individual vaccine components for check of stability and purity
RIA	• Binding occurs in native conformation • Low-concentration samples can be analyzed • High sensitivity antibody used at limiting dilution that conserves reagent • Can be plate-based for higher throughput (e.g., scintillation proximity assays)	• Requires radioactive labeling for detection • Shorter half-life of some radioisotopes requires periodic preparation of the tracer • Hazardous waste	• Protein identification (e.g., hormones) • Specific protein concentration analysis in complex samples
SRD	• Precise • Simple setup	• Semiquantitative • Low precision • Low sensitivity	• Vaccine release test
Precipitation	• Low equipment cost	• Subject to interpretation • Slow • Poor sensitivity (µg range)	• Vaccine identification
Agglutination	• Rapid • Low equipment cost	• Subject to interpretation • Slow • Low specificity because of interfering substances	• Vaccine identification

KEY CONSIDERATIONS IN ITM DEVELOPMENT

The goal during method development is to produce an accurate assay that is practically feasible and possesses an acceptable degree of intra- and inter-assay precision. To minimize the overall imprecision, the sources of variability should be identified and minimized.

Reagent Selection

Immunoassays are subject to several sources of interference such as cross-reactivity, endogenous interfering substances, buffer matrices, sample components, exposed versus masked epitopes, conformation changes in the antigen of interest, and other factors. Hence, during method development, analysts must identify possible sources of interference both to develop a robust method and to aid future troubleshooting.

Cross-reactivity is a major obstacle during immunoassay development. This arises when the specificity of an antigen–antibody reaction is compromised by the cross-reactivity binding of structurally similar molecules with the reaction binder. Some common

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examples are protein isoforms, degraded analyte entities, molecules of the same class, precursor proteins, metabolites, etc. Cross-reactivity can be minimized by rigorous reagent characterization and selection.

Reagents used in ITM applications generally fall into one of two categories: critical reagents and noncritical reagents. Critical reagents are specific and unique to the particular ITM or reagents that are intolerant of very small changes in composition or stability. Examples of critical reagents generally include assay-specific antibodies and reference or method calibration standards. Equivalence in the assay format must be established before replacement with a new lot. Noncritical reagents are those that can vary to some degree in composition without adversely affecting ITM performance. Reagents are often assumed to be noncritical (e.g., buffers, water quality, blocking buffer, or substrate) but later may be identified as critical components if assay ruggedness fails and troubleshooting of ITM reagents begins. ITM-specific reagents, including vendor and catalog number, should be defined in test procedure documents.

Antibody selection is critical for development of a successful immunoassay because it defines the assay's specificity and sensitivity. Furthermore, during antibody generation, analysts should ensure that the immunization protocols support the end use of the antibodies. For some applications a more specific antibody can be generated by the selection of a small and specific immunogen and affinity purification of the antibody, resulting in highly defined epitope coverage. In other applications it may be critical to ensure broad coverage of the different available epitopes on the molecules of interest, and a polyclonal antibody (pAb) pool may be the best choice. Currently, monoclonal antibodies (mAb) are preferred for some applications for the detection of single analytes because of their high specificity, lot-to-lot consistency, and indefinite supply. Compared to polyclonal antibodies, mAb have a higher initial cost to produce, but for these applications, the advantages generally outweigh the initial cost. Other applications may require more comprehensive epitope selection to ensure that subtle changes in the molecule(s) do not prevent recognition of the entire antigen, and thus a pool of monoclonal antibodies, or a pAb pool, would be the preferred choice. The latter are widely used for detection in a complex mixture of analytes (e.g., host-cell proteins). Similarly, immunoassays may use two distinct epitopes on an antigen—one for capture and the other for detection—which greatly reduces cross-reactivity. Another approach to minimize cross-reactivity is to purify the antigen before immunoanalysis. Variations in incubation temperature and time can affect the reaction kinetics of antibody interactions with similar yet different antigens. Thus this property should be optimized to increase the specificity of antigen–antibody interactions.

Development of Immunoassays

Development is an important stage in the establishment of a suitable ITM. During development of an ITM, analysts explore various settings of assay parameters and interactions between parameters to identify conditions under which the assay will consistently produce reliable results using minimal reagents, effort, and time. In Quality by Design terminology, the "possible operating space" is the collection of settings of assay parameters explored, and the "design space" refers to the conditions under which the assay performs well. The necessary performance properties of the ITM (precision, accuracy, specificity, etc.) required depend on the intended use(s). During ITM development, analysts should consider the following:

- Antigen–antibody ratio;
- In sandwich immunoassays, the ratio of capture antibody to detector antibody;
- Antigen–antibody reaction kinetics in the sample matrix (antigen–antibody binding generally is not linear);
- Selection of the standard (full-length antigen for the standard or just a small portion of the antigen containing the antibody-binding epitope, among other considerations); and
- Matrix effects.

The use of design of experiments (DOE) is strongly recommended, and different DOE methods may be appropriate in each stage of development. Early in development, screening designs are particularly useful (generally two-level geometric fractional factorial designs). After screening (with a modest number of factors to study), full factorials or response surface designs are often appropriate. As development activities shift to qualification (ideally, if not typically, as the focus shifts to robustness), robust response surface designs often are a good choice. During qualification or validation, analysts may find it practical to simultaneously study robustness to assay operating conditions (using a small geometric fractional factorial) and validation parameters such as precision (via nested or crossed designs for random factors associated with repeatability, intermediate precision, and reproducibility).

Experiments that assess dilutional linearity and components of specificity, including matrix effects, usually involve construction of spiked samples. Although spiking often is performed in a dilution matrix, spiking a collection of actual samples or mixing actual samples is an important component of demonstrating robustness of dilutional linearity and components of specificity to the sample and matrix components.

Reagent Considerations

A procedure for qualifying reagent sources and vendors (including audits), ordering, receiving, and disposing of commercial reagents and consumables should be outlined in a standard operating procedure (SOP). The preparation of internal reagents must be documented in a manner that allows reconstruction. Commercial and internally prepared reagents must be labeled with identity, concentration, lot number, expiration, and storage conditions. The stability and assignment of expiration dates for internally prepared reagents often are based on available literature and scientific experience, but analysts may need to confirm these empirically. An SOP for extending expiration dating of critical reagents is recommended. In addition, analysts should implement a mechanism for reagent tracking and linking lot numbers to analytical run numbers. Unacceptable reagent performance is detected by tracking QC samples. Shifts in QC samples should prompt a review of analytical runs and changes in reagent lot numbers or review of possible deterioration of critical reagents. To avoid such shifts, analysts can cross-validate critical reagent lot changes.

The impact of collection and storage containers on analytical performance often is overlooked. When defining the stability and expiration of in-house reagents, analysts should record information about the storage container vendor, catalog, and lot number. The importance of a suitable reference standard and its characterization cannot be overemphasized for ITMs for

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biological products. Because of their inherent complexity, reference and calibration standards of macromolecular biologics often are less well characterized than are conventional small-molecule drug reference standards. If the calibration standard represents a mixture of different antigens (e.g., host-cell proteins), it should be shown to be representative of the antigen profile in the samples being tested. Consistency in ITM results depends on the availability of a suitable representative reference standard material.

VALIDATION

Analytical validation involves the systematic execution of a defined protocol and prespecified analysis that includes prespecified acceptance criteria. A validation demonstrates that an analytical method is suitable for one or more intended uses [see *Validation of Compendial Procedures* ⟨1225⟩, *Biological Assay Validation* ⟨1033⟩, and ICH Q2(R1)]. Qualification may involve similar or identical experiments and procedures as validation, but qualification does not require prespecified protocols, analyses, or acceptance criteria. In certain situations (e.g., use of a commercial kit), assay development may not be required before qualification. General information chapter ⟨1225⟩ discusses which assay performance characteristics must be examined during validation for four primary categories of intended uses. For example, analytical procedures that quantitate major bulk drug substances or active ingredients may not require validation of the detection and quantitation limits but do require validation of accuracy, precision, specificity, linearity, and range.

System Suitability or Assay Acceptance Criteria

The purpose of system suitability or assay acceptance criteria is to ensure that the complete system—including the instrumentation, software, reagents, and analyst—is qualified to perform the intended action for the intended purpose. All processes should be controlled by well-defined SOPs that ensure consistency, reduce errors, and promote reproducibility of laboratory processes. Training files for all personnel should be contemporaneous and should include some demonstration that analysts are qualified to perform the method and the specific ITM.

Instrument and software qualification begins with a definition of the design qualifications, including a risk assessment and gap analysis that identify potential threats to the collection, integrity, and permanent capture of ITM data. Qualification also includes installation qualifications (IQ) and operational qualifications (OQ). Purchased commercial instrument validation packages may require modification to meet the intended use at each facility. Instrumentation and software should be continuously monitored for acceptable functionality by performance qualification (PQ) and software validation test script reviews. Routine instrument maintenance is performed according to the manufacturer's recommendations, and additional maintenance may be required based on specific needs in the working environment. A complete history of routine and nonroutine instrument maintenance should be archived for each instrument. Software updates should be handled with change control and typically require additional validation. Adherence to 21 CFR 11 should be maintained.

To ensure robustness, establish a defined process for implementing new ITMs in the laboratory. Control documents should be in place, including method validation plans containing a priori method acceptance criteria and validation reports for the establishment of a new ITM. Well-written analytical test method documents are needed to ensure reconstruction of analytical results and to minimize laboratory errors.

Analytical test methods should include acceptance criteria for critical aspects of the assay, including the performance of the calibration curve, quality controls, agreement between sample replicates, procedures for repeat sample analysis, and identification and treatment of outliers, when applicable. Furthermore, an SOP should be implemented for unexpected event investigation and resolution.

DATA REPORTING

Units of Measurement

Quantitative ITMs generate test sample data with an estimated concentration based on a calibration curve fit to reference (or standard) samples using an appropriate mathematical model. When determining the amount of analyte in a manufacturing process, analysts often express the unit of measure in terms of mass of analyte per volume of solution (concentration) or mass of analyte per mass of product (e.g., parts per million). Depending on the nature of the measured analyte, the degree of measurement standardization, the geographic region, and the history of the method, analysts may express concentration in terms of weight per volume, mole per volume, or weight of analyte per weight of product. In some circumstances, concentration may be converted to an activity unit of measure in which the analyte mass is assumed to be 100% active. In certain circumstances, qualitative analysis using a predetermined cut-off value may be an acceptable alternative to quantitative methods.

Immunoassay Data Analysis

ITMs employ calibration curves prepared with reference standards of known (nominal) concentrations and are included in every bioanalytical method. This helps control variation associated with repeatability, intermediate precision, and reproducibility and permits the estimation of results for unknown test samples. Common simple statistical analyses assume that the (possibly transformed) data are normally distributed, have constant variance, are independent, and that an appropriate model has been used. For many assays, one or more of these assumptions may be inappropriate. Analysts should assess these assumptions using a substantial body of data (typically tens of assays). When these assumptions are not reasonable, the analysis becomes more complex.

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Calibration curves generally are characterized by a nonlinear relationship between the mean response and the analyte concentration and typically are plotted in a log-linear manner with the (possibly transformed and/or weighted) response variable (ordinate) plotted against the nominal calibrator concentration (abscissa) in log scale. The resulting curve that encompasses the assay's validated range is inherently nonlinear and often has a sigmoid shape with horizontal asymptotes at very low and high concentrations of analyte. Competitive ITMs have a negative slope, and noncompetitive ITMs are characterized by a positive slope. The analyte concentration in a test sample is estimated by inverse regression against the calibration curve. The final result often is obtained after multiplication of the estimated concentration in the assay by a dilution factor that is required to yield a response within the ITM's quantification range.

Under the guidance of a qualified biostatistician, analysts can implement outlier tests in controlled documents that permit the exclusion of spurious sample results. A well-defined procedure should be in place regarding how to identify, repeat, and report outliers. Outlier tests and interpretation of results are described in *Analytical Data—Interpretation and Treatment* ⟨1010⟩. Test results that fall outside of their predefined specifications or acceptance criteria should be evaluated by an out-of-specification investigation to identify a root cause.

Trending

A quality system includes monitoring of ITM performance by collection and review of ITM performance characteristics. Trending may detect shifts in assay performance that may be related to events such as assay reagent lot changes, addition of new analysts, shifts in environmental conditions, and others. SOPs, study protocols, analytical test methods, and decision flow charts are recommended to strictly define the handling, use, editing, rejection, acceptability, and interpretation of calibration data and test sample results for ITMs. It is not uncommon to have several raw data reviews, including peer, QC, and quality assurance review. Analysts must be able to distinguish such analytical issues from true changes in the measured analyte caused by changes or errors in the manufacturing process that have affected the product. Two of the most important outcomes of proper trend monitoring are detecting potential problems before they occur and identifying areas for corrective and/or preventive action. General information chapters *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and *Biological Assay Validation* ⟨1033⟩, as well as the statistical literature, contain guidance for various trending methods. Several ITM performance characteristics could be considered for monitoring. The most common trending value is evaluation of QC samples. Ideally, one or more QC sample is available for long-term trending in sufficient quantity and with demonstrated stability so that quality aspects can be assayed in every run and across multiple manufacturing lots. As the long-term QC sample is depleted or expires, crossover comparison and establishment of a new long-term QC sample should be completed. Systematic review of QC data across assays assists in troubleshooting failed ITM runs, providing confidence in the evaluation of spurious results, and controlling the introduction of replenished assay components that may not perform exactly like previous reagents.

Other ITM performance characteristics that may be monitored include calibration curve response variables, curve fit parameters, assay background, and comparison of in-study QC data with validation data.

Tracking

Regulatory agencies have strict requirements about maintaining the identity and integrity of both samples and data. A quality process driven by SOPs must be implemented to ensure the correct identity and integrity of test and reserve samples. Ideally, a bar code system should be used to track the collection, identity, location, chain of custody, number of sample freeze/thaw cycles, storage temperature, and length of time that a sample is stored. This information should be captured and should be auditable from the time of collection to disposal (or sample depletion). The ability to track the sample history permits reconstruction of the events leading to generation of a data result. This information is used by regulatory agencies to ensure that the proper procedures were followed and by internal auditors to ensure that pre-analytical sample handling did not compromise study data. In addition, sample tracking allows a mechanism for ensuring that the analyte measurement occurred within the demonstrated window of stability for that analyte.

The final result generated from a bioanalytical laboratory is a number that represents an analyte measurement in a test sample. The steps necessary to generate that data and preserve it in a report are numerous and are susceptible to error. Therefore, quality systems must be in place to minimize data errors. Errors may be introduced by test sample misplacement or identification, incorrect data reduction, miscalculations, transcription errors, omissions, and other factors. Ideally, validated software and laboratory information management systems are used when possible to generate, transfer, and archive data. Typically, redundancy checks are built into automated processes by visual data review of at least 10% of the data-transfer processes. In the absence of validated electronic transfer, all data should be reviewed by at least one reviewer. As with sample tracking, data generation, manipulation, and storage should be reconstructible. In addition, all data should be backed up using a format that is stable. Plans should be in place to update archived data so that, as technology changes, archived data can still be retrieved. Regulatory agencies require that raw data be available for various lengths of time after the completion of a study or regulatory filing. Finally, data must be secure from corruption, alteration, or access by unauthorized personnel.

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⟨1103⟩ IMMUNOLOGICAL TEST METHODS—ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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INTRODUCTION

Immunological test methods (ITMs) utilize bindings between an antigen (Ag) and antibody (Ab). (See *Appendix 1: Abbreviations* for a complete list of acronyms used in this chapter.) Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used ITMs for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term "ELISA" is used here in a broader sense and includes enzyme immunoassays (EIAs), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

This chapter provides analysts with general information about principles, procedures, experimental configurations, assay development, and validation for solid-phase ITMs like ELISA and can be used for the other immunoassay variations mentioned above. The chapter also covers reference standards and controls used for immunoassays. The information can be adapted to the specific procedures of a monograph. This chapter does not cover immunoassays for the measurement of immune responses to product in animals or humans (e.g., serological or cellular assays), non-immunoassays (e.g., receptor-ligand interactions), or other related approaches.

The chapter is part of a group of general information chapters for ITMs (*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩, and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩), and also is related to the general information chapters for bioassays (*Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩).

Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly (as with an analyte that has enzymatic properties or is directly labeled with an enzyme) or indirectly (by means of an enzyme-linked antibody that binds to the immunosorbed analyte). Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This standard should be an appropriate, preferably homologous, reference or calibration material that is representative of the analyte(s) of interest. The power of immunoassays has been demonstrated by the variety of procedures that have evolved, including alternative solid surfaces such as beads of different sorts, various plastics in plates of different configurations, and alternative detection methods, e.g., chemiluminescence and fluorescence. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

Basic Principles

The essential steps of an ELISA can be broken down as follows (see *Figure 1*):

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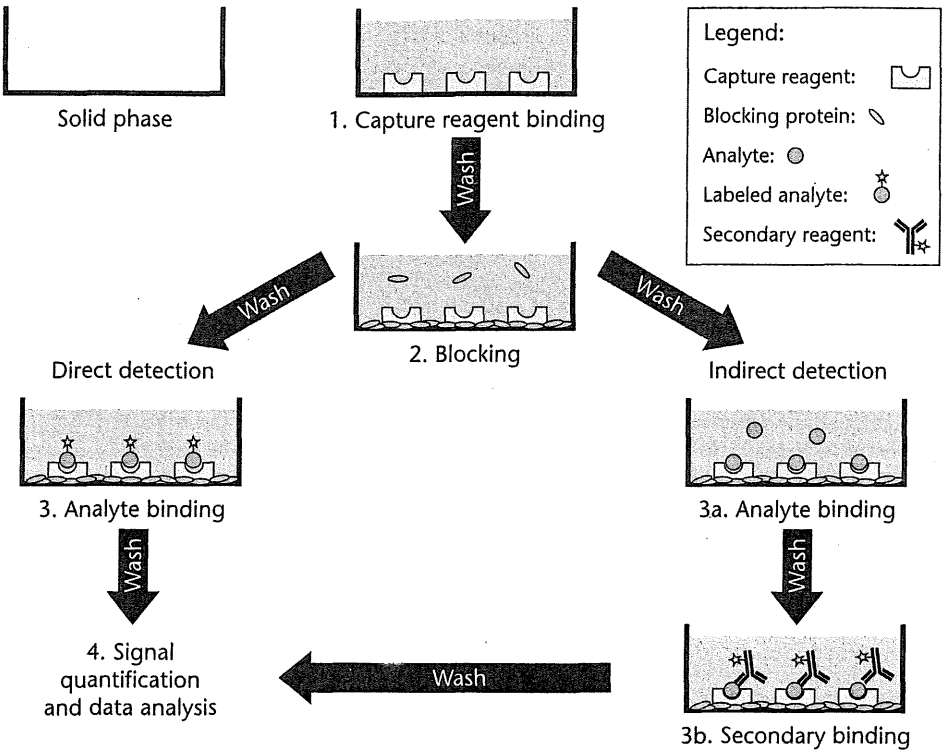

Figure 1. Essential steps for performing an ELISA.[1]

1. Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;
2. Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;
▲3 or▲ (USP 1-Dec-2019) 3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte.
▲3.▲ (USP 1-Dec-2019) Direct detection occurs when the analyte has enzymatic activity or has been linked to a detector molecule (e.g., enzyme); or
3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte (*Figure 1*, step 3a). Indirect detection occurs when the analyte is detected by the addition of a secondary enzyme-labeled reagent (*Figure 1*, step 3b); and
4. Quantification of the analyte by addition of a substrate suitable for the detector used [e.g., 3,3′, 5,5′-tetramethylbenzidine (TMB)], followed by comparison of the test sample to the reference standard.

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ASSAY DESIGN

▲Four▲ (USP 1-Dec-2019) general categories of ELISA are described in *Table 1* and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

[1] Capture reagent binding, blocking, analyte binding, detector antibody binding, and analysis are the five basic steps in an ELISA. Capture reagent binding, blocking, and analyte binding steps are each followed by a washing step to remove unbound reagents before the addition of the next reagent. Before analysis an appropriate substrate is added, followed by measurement of the substrate by appropriate equipment for detection. Quantitation of unknowns takes place by comparison with a standard curve.

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Table 1. Representative ELISA Types

ELISA Type	Required Reagents	Attributes	Disadvantages
Direct detection	• Capture analyte[a] • Labeled primary antibody specific for antigen	• Rapid because only one antibody is used • Uses less reagent • Analyte is immobilized	• May modify the conformation of the analyte • Sensitive to matrix and adjuvant components • Not commonly used • Poor sensitivity
Indirect detection	• Capture analyte[a] • Primary antibody specific for antigen • Labeled secondary detector antibody that binds to the primary antibody	• Versatile because a variety of primary antibodies can be used with the same secondary detector • Improved sensitivity because of signal amplification • Analyte is immobilized	• Longer because of more incubation and washing steps
Competitive	• Analyte[a] can be used as a capture reagent or can be labeled with a detection label • Antibody specific for analyte can be used for capture or labeled for detection • Labeled secondary antibodies to bind to primary antibody if an indirect format is used	• Good for assessing antigenic cross-reactivity • Appropriate for smaller proteins with single epitopes • Requires only a single antibody • Analyte in solution competes for binding to primary antibody	• Format difficult to troubleshoot • Limited dynamic range
Sandwich	• Primary capture antibody specific for analyte • Sample solution containing analyte[a] • A different primary enzyme-antibody conjugate specific for analyte	• Improved sensitivity • Good for quantitative assays for larger multi-epitope molecules • Analyte measured in solution	• Requires relatively large amounts of pure or semipure specific antibody • Not suited for smaller proteins that may have only a single epitope or a few closely spaced epitopes

[a] This reagent can be either purified or partially purified. The terms "analyte" and "antigen" are used interchangeably when describing ELISAs.

Direct ELISA

DIRECTLY LABELED ANTIBODY

In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked [*Figure 2* (A)]. Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away, followed by the addition of an appropriate substrate for the detector used.

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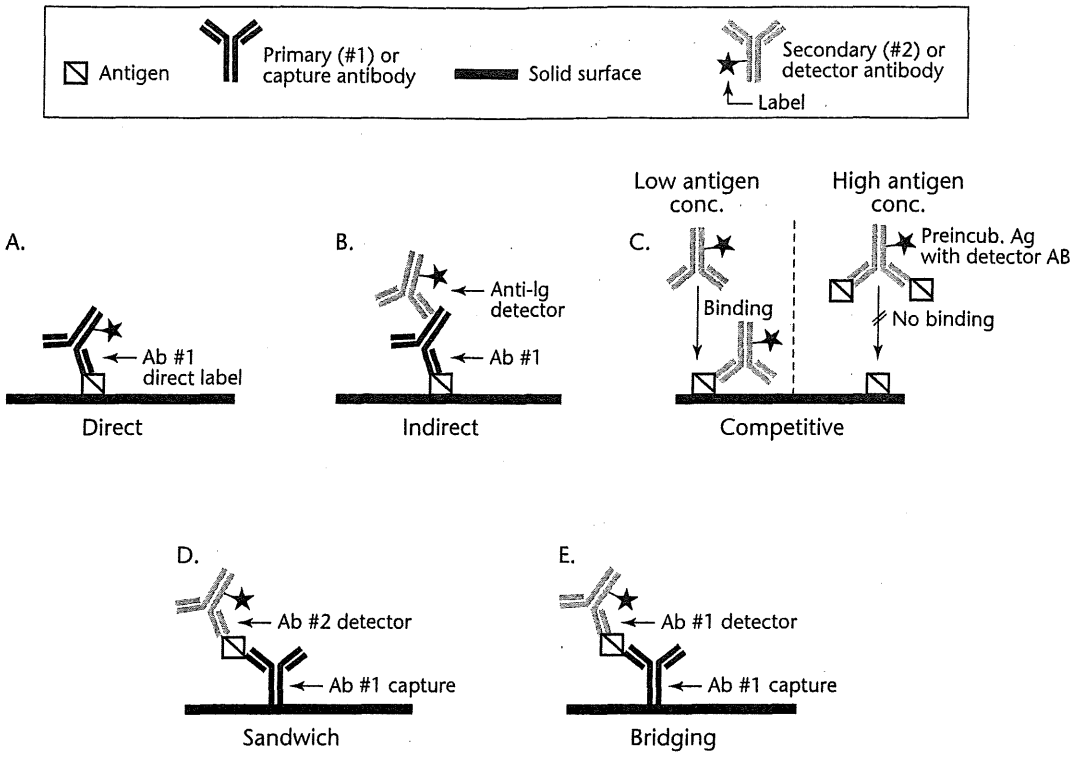

Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.[2] [Ab = antibody; Anti-Ig = anti-immunoglobulin; Ag = antigen (or analyte); Conc = concentration; Preincub = preincubation]

DIRECTLY LABELED ANTIGEN

This assay is similar to that using a directly labeled antibody, except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added [*Figure 2* (B)]. After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a *Direct ELISA*, thereby increasing assay sensitivity.

Competitive ELISA

DIRECT ANTIBODY COMPETITIVE ELISA

This assay is used to detect or quantitate soluble antigens [*Figure 2* (C)]. It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase (HRP), alkaline phosphatase (AP), ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen–antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., colorimetric, electrochemiluminescence, fluorescence, or chemiluminescence) is measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

[2] The type of ELISA format depends on the availability of reagents, the intended purpose of the assay, and the physicochemical characteristics of the analyte of interest. For a *Bridging ELISA*, the capture and detector antibodies recognize the same epitope, and therefore the target antigen must have at least two epitopes available for binding.

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DIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

INDIRECT ANTIBODY COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

INDIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antigen Competitive ELISA*, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

Sandwich ELISA

DIRECT SANDWICH ELISA

In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added [*Figure 2* (D)]. After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

INDIRECT SANDWICH ELISA

Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detection could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

BRIDGING ELISA

This subset of *Sandwich ELISA* assays often uses a single antibody for both capture and detection [*Figure 2* (E)]. If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g., an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the EC_{50} and require a wider dose range.

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PROCEDURES

Solid Phase

Solid phases are available in a variety of forms (e.g., membrane, plate, or bead) and chemistries [e.g., nylon, nitrocellulose, polyvinylidene fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface]. The selection of the solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

COATING THE SOLID PHASE—IMMOBILIZATION OF CAPTURE REAGENT

Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye's color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 µg/mL are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 µL. Coating duration, temperature, and buffers are discussed separately below. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

MICROTITER PLATES

The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require optimization of buffer pH based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half-volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

COATING TIME

Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

COATING TEMPERATURE

Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen. Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

BUFFERS

Buffers used for diluents, coating, blocking, and washing plates can affect overall assay performance. Buffer components can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

Diluent: Buffers [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer.

Coating buffers: Coating buffers should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffers include 50 mM carbonate, pH 9.6; 20 mM tris-hydrochloride (tris-HCl), pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer depends on the nature of the individual antigens and should be determined empirically.

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Blocking agents and buffers: A blocking agent is a compound (e.g., protein or detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the dilution buffer. The choice of blocking agent should be governed by the nature of the capture reagent, plate, coating buffer, test sample diluent, and related factors. If any of these parameters changes, a change in blocking agent may be needed. Commonly used blocking agents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITM.

Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. ▲A sample loading pattern should be included in the test method procedure. For reproducibility and accuracy of results, consistency between the wells of the ELISA plate is very important. This can be achieved by using replicates; however, as mentioned above, care should be taken to avoid replicates in adjacent wells. A common way of avoiding the edge effect is not to use the edge wells at all. Additionally, plate edge effects can also be avoided by reducing assay time, using a low evaporation lid, or sealing the plate with a clear or breathable sterile tape.▲ (USP 1-Dec-2019) Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

PIPETS

Single, multi-channel, and robotic pipets with set or fixed volumes are available. The type and accuracy of pipets should be evaluated for each application. Regular maintenance and professional calibration of pipets should be performed and documented.

PIPET TIPS

A variety of pipet tips are available, some of which are specific to the type of pipet. The type and accuracy of the pipet tip, particularly related to the viscosity and nonspecific binding of the materials, should be evaluated for each application.

Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and detection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in wash buffer, and adding wash buffer with a multi-channel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multi-well washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer left in the well. Incorrectly programmed or maintained, as well as incompletely cleaned, automatic washers can cause assay variation and elevated assay background.

Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

Blocking Conditions and Nonspecific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1. Ionic or hydrophobic interactions occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.
2. Immunological interactions occur when binding is mediated by unintended antigen–antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed

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to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

The choice of blocking agent (examples are found in *Blocking agents and buffers*) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffers that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer in place of sample. Control wells also can be useful as part of system suitability testing.

Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article's properties and/or introduce further variability that results in biased measurements. Samples, standards, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

Detector Antibodies

Depending on the ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA [*Figure 2* (A and C)], after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats [*Figure 2* (B, D, and E)], the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the conjugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate controls.

The most commonly used labeling enzymes for conjugating to antibodies include AP, HRP, and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. *para*-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, *o*-phenylenediamine dihydrochloride (OPD), and [2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] (see *Table 2*). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see *Table 2*). Well-known fluorescent substrates for galactosidase include 4-methylumbelliferyl galactoside (MG) and nitrophenyl galactoside (NG). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

Table 2 also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay's purpose and the requirements of the assay.

Table 2. Enzyme Conjugates and Substrates

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Reader	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP, HRP	pNPP TMB OPD ABTS	Spectrophotometer	• Robust • Economical • Reagent availability	• Less sensitive

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Table 2. Enzyme Conjugates and Substrates *(continued)*

Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP	CSPD	Luminometer	• Wide assay dynamic range • Lower coating concentrations • More sensitive • Rapid signal generation	• Requires special plates • Costly
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase	MG NG	Fluorometer	• Rapid • Sensitive	• Requires special plates • Costly • Interference by excipients

ASSAY DEVELOPMENT AND VALIDATION PLAN

Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated (see, for example, guidance contained in ⟨1032⟩).

SOURCE

The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances, it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

PURITY

In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

SPECIFICITY

The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific binding or no cross-binding to off-target molecules in complex test materials.

STABILITY

The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

1. Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
2. Understand the performance goals for the assay system.
3. Develop the assay to the point that there is a detectable concentration response curve.
4. Perform method development/robustness testing.
5. Prepare the reference/calibration standard and control and assess stability.
6. Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
7. Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
8. Validate the assay.
9. Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
10. Monitor assay performance.

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During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs.

Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

Assay Validation

Assay validation is executed according to guidances from appropriate regulatory bodies [e.g., International Council for Harmonisation (ICH) Q2] to demonstrate that the particular test used for an analyte is appropriate for its intended use. More information about assay validation can be found in *Validation of Compendial Procedures* ⟨1225⟩ or ⟨1033⟩ if the ELISA is used as a surrogate potency assay. See *Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis*.

Change to read:

DATA ANALYSIS

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a nonlinear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration (IC_{50}) or effective concentration (EC_{50}), estimation of the amount of a sample that yields the same response as the EC_{50} (or IC_{50}) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard. More extensive guidance about statistical methods for potency analysis is given in ⟨1032⟩ and ⟨1034⟩.

In general, ELISA curves are characterized by a nonlinear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

Basic Statistical Analysis

Basic statistical methods are not detailed here. *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses important fundamentals, including data handling; computation of means, standard deviations, and standard errors; detection of and methods to address nonconstant or nonnormal variation; detection of and management of outliers; and procedures for and interpretation of statistical tests and confidence intervals. The concepts behind validation, goals, designs, analysis, and practical methods for validation are described in ⟨1010⟩, ⟨1225⟩, and ⟨1033⟩. *Design and Analysis of Biological Assays* ⟨111⟩ contains guidance on combining results from independent assays, ▲outliers, and confidence intervals.▲ (USP 1-Dec-2019)

Nonlinear Statistical Analysis

Nonlinear calibration for immunoassays draws on many sources for statistical design and analysis. These include methods for assessing and addressing nonconstant variance, designs and analysis methods for experiments with complex structures, and validation. The concepts behind linear calibration design, analysis, and inverse regression apply in nonlinear calibration, and professional statisticians can help apply these appropriately.

Reporting Results

Reported estimates of concentration should be understood as having an associated confidence interval based on the results of the validation. The reported value or estimate used to describe a sample can be based on a combined result from multiple assays.

Change to read:

APPENDICES

Appendix 1: Abbreviations

Ab: Antibody
ABTS: 2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
Ag: Antigen
Anti-Ig: Anti-immunoglobulin
AP: Alkaline phosphatase
BSA: Bovine serum albumin
CSPD: Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate

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EIA: Enzyme immunoassay
ELISA: Enzyme-linked immunosorbent assay
HRP: Horseradish peroxidase
Ig: Immunoglobulin
ITM: Immunological test method
MG: 4-Methylumbelliferyl galactoside
NG: Nitrophenyl galactoside
OPD: *o*-Phenylenediamine dihydrochloride
PBS: Phosphate-buffered saline
pNPP: *para*-Nitrophenyl phosphate
PVDF: Polyvinylidene fluoride
TMB: 3,3′,5,5′-Tetramethylbenzidine

Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis

	Analytical Data—Interpretation and Treatment ⟨1010⟩	Design and Analysis of Biological Assays ⟨111⟩	Validation of Compendial Procedures ⟨1225⟩	Biological Assay Chapters ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩
Means	X	—	—	—
Standard deviations	X	—	—	—
Standard errors	X	—	—	—
Non-normality	X	—	—	X
Nonconstant variance	X	—	—	X
Outliers	X	▲X▲ (USP 1-Dec-2019)	—	X
Tests	X	—	—	—
Confidence intervals	X	▲X▲ (USP 1-Dec-2019)	—	X
Validation	—	—	X	X
Combining results from multiple assays	—	X	—	X

⟨1104⟩ IMMUNOLOGICAL TEST METHODS—IMMUNOBLOT ANALYSIS

INTRODUCTION

Definition and Scope

The chapter is part of a group of general information chapters for immunological test methods (*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Enyzme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩) that provides analysts with general information about principles, method development, method validation, and data evaluation for immunoblot analysis. Immunoblot analysis is defined as any method in which an antibody is used for detection of one or more analytes (e.g., proteins, polysaccharides) that has been transferred to a test membrane surface. Immunoblot methods are typically classified by whether electrophoretic separation occurs as a part of the immunoblot procedure. Electrophoretic separation is based on molecular weights and charge differences of a population of molecules. See *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩ for a detailed description of electrophoretic separation methods. An example of immunoblot analysis involving electrophoretic separation is the Western blot, which was first described in the scientific literature in the late 1970s. Another approach for immunoblot analysis is to perform molecule detection using an antibody without prior electrophoretic separation. Examples of this nonelectrophoretic type of approach are the slot or dot blot (slot/dot).

The scope of this chapter includes only those methods in which an antibody is used for the detection of a molecule bound to a membrane. Therefore this chapter does not discuss procedures that use nonimmunological means of detection.

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ASSAY SELECTION

Nonelectrophoretic Assay (Slot/Dot Blot)

The slot/dot blot method is a simplified, nonelectrophoretic method in which a mixture containing the analyte(s) for detection is first applied directly to the membrane using a vacuum manifold machined to contain regularly spaced rectangular slots. The slot/dot blot method is faster and simpler because there is no electrophoretic separation of the multiple, individual analytes that may be present in the mixture. For these reasons, it can be readily adapted for automated analysis of multiple samples, for which a number of systems are commercially available, but it offers no information about the molecular weight and only limited information regarding the quantity of sample. Although it can be set up in a quantitative format, the method usually is used to produce a qualitative result, e.g., confirming identity by demonstrating the presence or absence of specific antigens by means of an immunocomplex detection system. After the analytes are bound to the membrane and unbound sites are blocked, analysts use a detector antibody to determine the presence or absence of the analyte or analytes of interest. The uniform shape of the slot blot and its greater surface area for analyte binding make it better suited than the dot blot for quantitative applications and analysis by densitometry.

Electrophoretic Assay

Electrophoretic blotting methods (commonly called Western blots) are widely used for analyzing mixtures of proteins. The Western blot is a powerful tool to study the identification, relative concentration, relative molecular weight, and posttranslational modifications of specific proteins. In Western blots, the proteins of the sample are separated using gel electrophoresis. Protein separation may be based on molecular weight alone or on isoelectric point (pI) and molecular weight. Proteins migrate either in one dimension (1D) or in two dimensions (2D) through a gel. When proteins are separated by their molecular weights, the smaller proteins migrate faster and separate according to molecular weight. When analysts use a 2D gel, proteins are separated according to pI in the first dimension, and then according to their molecular weights in the second dimension. After separation, the proteins are transferred to a membrane, the membrane is blocked to avoid nonspecific binding of subsequent assay reagents, and the protein of interest is detected using specific antibodies.

A bound antibody can be detected by different methods, including colorimetric detection, fluorescent detection, chemiluminescent detection, and radioactive detection. Upon detection of the protein(s) of interest, immunoblot quantitation can be indirectly performed by densitometry.

1D ELECTROPHORESIS

In 1D electrophoresis, individual proteins or groups of proteins are separated by molecular weight for further analysis by Western blot. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins migrate in response to differences in electrical charge through a 3D network of fibers and pores. The network is formed as the bifunctional bisacrylamide, or other cross-linker, cross-links adjacent polyacrylamide chains to form a gel (see also *USP* general chapter ⟨1056⟩). The combination of gel pore size and protein characteristics determines the migration of proteins. Separated proteins are detected subsequently by Western blot analysis using antibodies specific to the target proteins. By means of Western blot analysis, a test sample can be compared to a standard, and the appearance of degradants and impurities specifically related to the target proteins can be monitored if the detection antibody can still recognize the altered forms of the protein. Although a high level of sensitivity can be achieved by this approach, separation of individual proteins at similar molecular weights may not be achieved. If analysts must probe individual proteins at similar molecular weights, 2D separations may be required.

2D ELECTROPHORESIS

In 2D electrophoresis, individual proteins or groups of proteins are separated in the first dimension by isoelectric focusing (IEF; charge) and in the second dimension by electrophoresis in the presence of SDS (molecular weight). Separating proteins this way allows information to be obtained not only about molecular weight, as in 1D gels, but also about the charge of a protein. Two-dimensional gels are a useful choice for resolving complex mixtures and for assessing protein antibody specificity (e.g., evaluation of host cell proteins).

Membrane, Reagent, and Detection Options

MEMBRANES

Generally, both nitrocellulose and polyvinylidine fluoride (PVDF) membranes are used for immunoblot methods. For cost considerations, nitrocellulose membranes are often preferred over PVDF membranes for slot/dot blots (or vacuum blotting), but due to their greater mechanical strength, PVDF membranes should be considered if stripping and reprobing are required.

BLOCKING REAGENTS

Following transfer or binding of protein to membranes, the unoccupied binding sites on the membranes must be blocked to prevent nonspecific binding of subsequent reagents. Most detection probes are proteins that also can bind to the membrane. Failure to appropriately block the membrane sites can result in nonspecific binding and high background. A number of blocking reagents are available, including gelatin, nonfat milk, and bovine serum albumin (BSA). Proteins should be unrelated to the antigens used in the study. Because these reagents often have lot-to-lot variability, they may require qualification. They must be evaluated with the detection system selected and optimized using that detection system for minimal background with no

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loss of signal. If the blocking reagent is derived from a biological source, it must not contain trace levels of the protein under measurement, because the latter can increase the background.

METHODS OF DETECTION

Immunological detection of analytes in any type of immunoblot can be direct or indirect. The choice of format depends on a combination of the level of sensitivity required and the quality of the antisera available. For identity or product detection, sensitivity usually is not critical, and direct detection via a conjugated antibody is commonly used, which often simplifies and shortens the time required to execute the method. Alternatively, indirect detection, usually by the use of a conjugated anti-species reagent, can be used to improve sensitivity. On some occasions, the analyte being detected is actually an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, antibodies specific for the antibody (e.g., anti-idiotypic antibodies) can be used.

Primary antibody: The primary antibody is selected based on its specificity for the analyte or protein. Although polyclonal anti-sera can offer a broad range of detection against a potentially large set of epitopes, an unwanted cross-reaction resulting in decreased specificity may occur. If this cannot be overcome by assay optimization, a monoclonal antibody or groups of selected monoclonal antibodies can be used. Monoclonal antibodies are often advantageous for long-term studies, because they yield a consistent supply of antibody against a specific epitope. The use of monoclonal antibodies directly limits the number of epitopes involved in the detection of the target. This must be evaluated for each application. The primary antibody may be directly conjugated or used in conjunction with a secondary antibody and an appropriate detection system. The optimal antibody concentration usually is considered to be the greatest dilution of antibody that results in a strong positive signal with minimal background. This must be optimized in conjunction with the block and detection system selected. The primary antibody should be qualified before assay use.

Secondary antibody: The secondary antibody typically is directed against the species of the primary antibody immunoglobulin (which is specific for the analyte, e.g., goat anti-mouse IgG). Enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) typically are linked to the secondary antibody, but other labels such as fluorophores or gold particles can be used for detection. If the secondary antibody is biotinylated, biotin–avidin–HRP or –AP complexes can be used for detection.

Detection enzyme and substrate: Once an immunocomplex containing the enzyme–conjugate reagent has formed, analysts add a suitable substrate to the assay. This reaction results in production of a colored precipitate or a fluorescent or chemiluminescent product that can be recorded, measured, and analyzed further. A broad range of detection options is available to best fit individual applications and intended uses. A number of these are described in ⟨1103⟩ and ⟨1102⟩, as well as in *Table 1*.

Table 1. Detection Reagents and Methods

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Detection	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP[a] HRP[c]	pNPP[b] TMB[d] OPD[e] ABTS[f]	Spectrophotometer	— Robust — Economical — Reagent availability	— Time-consuming — Less sensitive than other methods
Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP, HRP	CSPD[g]	Luminometer, photographic film (CCD[h] camera)	—Wide assay dynamic range — Very sensitive — Rapid signal generation	— Reproducibility can be challenging
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase, fluorescently labeled antibody	MG[i] NG[j]	Fluorometer (CCD camera with filters)	— Rapid — Sensitive	— Interference by excipients
Radioactive	Antigen is labeled with a radioactive isotope. Radiation is proportional to analyte concentration.	—	—	Scintillation counter	— Easy to quantitate — Rapid	— Safety risk with exposure — Radioactive waste

[a] Alkaline phosphatase.
[b] *para*-Nitrophenyl phosphate.
[c] Horseradish peroxidase.
[d] 3,3′,5,5′-Tetramethylbenzidine.
[e] o-Phenylenediamine dihydrochloride.
[f] 2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt.
[g] Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate.
[h] Charge-coupled device.
[i] 4-Methylumbelliferyl galactoside.
[j] Nitrophenyl galactoside.

METHOD DEVELOPMENT

Method development can proceed on the basis of the background information just provided. The scope of method development, and eventually method validation, are dictated by the purpose of the method. The purpose determines the format

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for the assay and other requirements for the test, and therefore, the purpose should be determined first. The following sections explore considerations for each method's purpose.

Intended Purpose of the Method

IDENTITY TESTING

In the case of identity tests, analysts want to detect the presence of a protein; therefore, demonstration of specificity is essential and required. For this purpose, analysts also control the quantity of protein in the sample. Thus, the limits of detection (LOD), limits of quantitation (LOQ), and other measures of quantity are not required attributes of the method. Examples include material identity assays that demonstrate the isotype of an IgG and, in some cases, demonstrate the specificity of an antibody in a method validation. If there is no interference from the matrix or potential cross-reaction with other materials present in the sample, then a simple slot/dot blot may suffice. If multiple proteins in the sample display immunoreactivity and must be distinguished from each other, another separation procedure must be used before blotting and immunostaining. The complexity of the proteins in the sample and the usefulness of the additional information gained using an electrophoretic separation help determine if a slot/dot blot can meet the needs of the test.

LIMIT TESTING

In other applications, analysts may want to show that an impurity has been removed to a level below toxicological concern. In many cases, a limit test is used when it is possible to say *yes* or *no* about the presence or absence of a protein below a predetermined level. This simplifies the development and validation of the method. With densitometry (scanning or imaging) equipment, the intensity of spots or bands can be determined relative to a standard curve, resulting in an estimate of concentration. An LOD should be determined to establish the appropriate limit threshold for the method. A dot blot may be suitable for either circumstance if the specificity of the antibody in the sample matrix can be demonstrated.

Another common purpose for an immunoblot is to show the presence or absence of a protein expressed from a culture. In this situation, analysts want to establish the identity of the protein by immunostaining, as well as verify that the protein has the expected molecular weight. This provides further assurance of no nonspecific interactions with other proteins in a complex matrix that generates the signal in the blot.

SPECIFICITY TESTING

Characterizing the specificity of the reagents for an ELISA impurity test or an immunoaffinity column also is a common immunoblot purpose. This is another form of an identity test in which the desired endpoint is demonstration of the specificity of binding between the antigen and the antibody. The result of the measurement is a demonstration of binding to a select group of the total protein population in the sample or binding to the whole population of proteins in the sample, as required for host-cell protein assays. In order to demonstrate the specificity of an antibody relative to a population of proteins, analysts typically must carry out electrophoretic or other separations. Showing, by means of immunostaining, that a protein of the right molecular weight or pI can be recognized by the antibody is a powerful demonstration both of specificity toward a given protein and the absence of binding to other proteins. In addition, having, within the same experiment, the appropriate positive control samples that are known to contain the protein, and the appropriate negative samples that are known not to contain the protein, makes a convincing argument for the specificity and selectivity of the antibody when analysts validate an ELISA method for protein impurities. Electrophoretic separations can be done in one dimension using either SDS–PAGE (for molecular weight) or isoelectric focusing (IEF; for isoelectric point) for a limited number of proteins with known molecular weights. Electrophoretic separations also can be done in two dimensions (e.g., IEF followed by SDS–PAGE) to show selectivity and specificity toward a more heterogeneous population of proteins. A 2D Western blot commonly is used to demonstrate the specificity of a polyclonal antibody candidate directed against a host-cell protein (HCP) antigen preparation before development of a quantitative ELISA for that purpose.

Assay Mode and Sample Introduction

After considering the critical elements required for each purpose of the method, the analyst can use this information to select the most appropriate assay mode. The main points to consider when developing an immunoblot method are shown in *Figure 1*. If appropriate, spotting samples on a membrane or applying by vacuum is the easiest and most convenient way to introduce a sample to an immunoblot membrane. However, low levels of nonspecific binding from multiple proteins can create additive nonspecific interference in dot blots or slot blots, resulting in background levels that appear to be the desired analyte.

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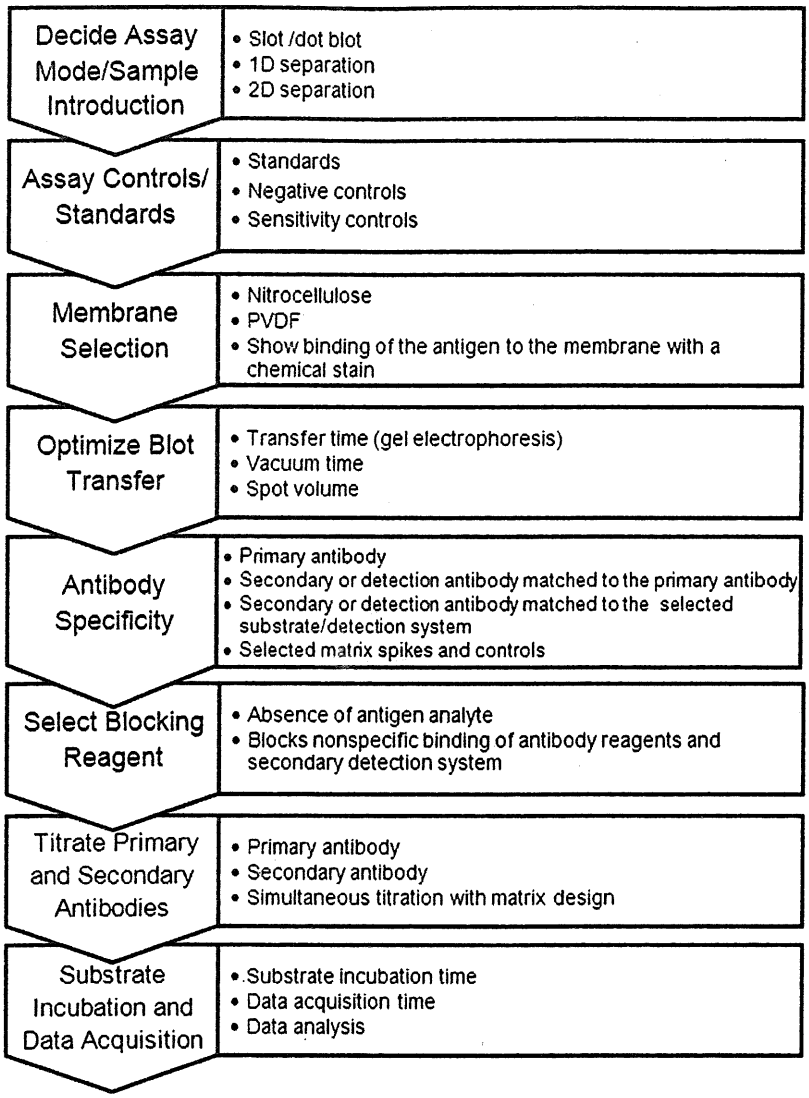

Figure 1. Method Development Flow Chart.

Electrophoretic separations, although time consuming, can be useful for separating and further distinguishing specific and nonspecific binding. Analysts must trade sensitivity for selectivity in going from a single dimension to two dimensions because of the further separation of immunoreactive species from a single band into multiple spots, as is the case with the heterogeneity seen in sialylated proteins or deamidated species.

Assay Controls and Standards

Controls and standards are selected based on the purpose of the assay and the information needed during development. Protein molecular weight markers can be used to obtain an accurate estimate of the molecular weight of immunoreactive species. The use of positive and negative controls is helpful for troubleshooting throughout the experimental design process. Standards or positive and negative controls can be used to assess system suitability and to establish method performance. A positive control can confirm appropriate protein migration and can confirm that membrane transfer has reached completion. A negative control is useful for assessing nonspecific interactions. A method sensitivity control near the LOQ can be used to measure the consistency of the method near the LOQ to evaluate changes in assay performance.

Membrane Selection

A membrane is selected based on the application and the protein being measured. Membranes with various pore sizes should be available and should be suitable for the molecular weight of the protein of interest to aid in appropriate transfer of different sizes of proteins. If a chemical staining method is known to work on a specific membrane with a specific protein, then it is advantageous to show that the protein binds to the membrane and is stained before analysts work on the immunostaining

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steps for the assay. Electrophoretic separations followed by transfer to a membrane should be optimized with chemical staining, e.g., with sensitive fluorescent stains or silver stains and at potentially higher loads before analysts work on lower load levels required for blot optimization. Stains such as Coomassie or colloidal Coomassie may not have sufficient sensitivity to detect a low level of impurities required for certain applications.

Optimize Blot Transfer

Analysts should optimize transfer times from the gel to the membrane. Larger proteins require more time for transfer than smaller proteins. Small proteins may be lost during long transfer times and can transfer from the gel all the way through the membrane and be lost on the other side. The density of the gel and gradient gels can result in nonuniformity of transfer from the top to the bottom of the gel. During transfer optimization, many method developers use multiple membranes in order to capture proteins that transfer through the first membrane. Chemical staining of both the gel and the membranes can provide useful information about the location of the proteins transferred from the gel to support, either extending or reducing the transfer time.

After they select the assay mode, analysts next investigate spotting of the antigen or transfer from a gel to the appropriate membrane at various relevant concentration levels. Levels of analyte above the concentration needed for a Western blot may be required at first to determine if transfer and recognition by the antibodies is possible. If the analyte is present in low concentrations, spiking may be necessary to show its location during transfer optimization. Because of the potential variability of immunostaining and transfer, a sensitivity control or several levels of controls should be incorporated into the method based on the analyte titration above the background level. This can be adjusted as method development progresses.

Antibody Specificity

Analysts should demonstrate antibody specificity early in immunoblot method development. If possible, they should test samples of the matrix without the analyte and should show an absence of response. In contrast, samples that contain analyte spiked into the matrix should show a positive response, demonstrating the specificity of the antibodies.

Analysts also should demonstrate the specificity of the secondary antibody conjugate or label. Control immunoblots with lanes or spots of primary antibody and control matrix samples containing the analyte as a negative control can show that the secondary antibody is binding to the primary antibody and not to proteins found in the matrix. Commercial sources for enzyme conjugates or fluorescent-labeled anti-species antibodies are readily available and normally are screened or affinity purified against the species of antibody being detected, which eliminates some of the early work needed to achieve the desired specificity. The secondary antibody or detection system must be matched with the detection equipment and the desired sensitivity of the assay, e.g., fluorescence, colorimetric precipitating substrates, or chemiluminescence.

Select Blocking Reagent

Replicate membranes can be blocked with previously described blocking agents as analysts select the most appropriate blocking reagent and the amount of time required to minimize background by means of subsequent primary and secondary antibody incubations. Analytes titrated at multiple concentrations on the membrane allow analysts to assess the amount of signal to the amount of noise (background) with various blocking reagents followed by immunostaining with the primary antibody, labeled secondary antibody, and substrate, if needed, for visualization. This titration also serves as the starting point for examining LOD and LOQ for limit tests and quantitative measurements. The LOD for immunoblots is determined by the level of nonspecific background relative to the specific signal from the analyte. As is the case with any other analytical method, if the background and signal are equal, there is no distinction between the signal and the noise.

Titrate Primary and Secondary Antibodies

Titrating the level of primary and secondary antibody from low to high dilutions can also, as with a blocking reagent, be used to select an antibody concentration that reduces background binding in the blank regions surrounding protein spots or bands, and can optimize the signal from the analyte. A matrix grid that varies the level of primary signal against secondary signal can be useful for optimizing the background, improving analyte signal, and reducing the consumption requirements for expensive antibody reagents.

Immunoaffinity chromatography against a highly purified antigen can be used to reduce the level of nonspecific interference for all of the immunological reagents used in an immunoblot. The method developer must be cautious that the selectivity, specificity, and affinity of the primary antibody are not lost in affinity purification because of high-affinity antibodies that remain on the antigen column or because of the destruction of antibody binding caused by elution conditions. For the secondary antibody, immunoaffinity-purified anti-species antibodies are available commercially with a variety of possible labels conjugated to the antibody.

Substrate Incubation and Data Acquisition

Analysts can optimize substrate development time for enzymes in order to minimize background and improve the LOD and LOQ. Excessive substrate development times for precipitating substrates can result in an intensification of the background level relative to the specific signal from the desired analyte. If the blot is agitated during substrate incubation, undesired swirling patterns of product from precipitating substrates can form. Too short an incubation time results in a less-specific signal, but too long a time can result in high background and poor resolution. Most enzyme conjugates have an optimum development time. Fluorescent labels and chemiluminescent labels have the advantage of acquisition by scanning instrumentation that can store

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data electronically and perhaps acquire image signals in an additive manner. Fluorescent labels have the added advantages that the signal is stable with time, numerous experiments for development time can be obtained with a single blot, and optimization of signal acquisition can be performed on a single blot.

PROCEDURES

Slot/Dot Blots

Using an appropriate slot/dot apparatus, analysts can make antigens of interest adhere to a suitable membrane (e.g., nitrocellulose) by gravity or vacuum filtration, followed by addition and incubation of antigen-specific antibodies that bind to epitopes on the antigens. Remaining binding sites on the membrane are blocked by the addition of nonspecific antigen (e.g., BSA), followed by probing of the antigen-specific antibodies with a detection system [e.g., protein A/G conjugated to HRP binds to the antibodies that then are visualized using a 4-chloro-naphthol (4-CN) peroxidase substrate]. Positive identification is the development of dots or bands on the membrane. A negative result remains white or exhibits faint bands that are considerably lighter than positive bands.

1D Immunoblotting

PREPARATION OF SDS–PAGE GELS

Analysts should choose an SDS–PAGE gel with a content of acrylamide–bisacrylamide suitable for the molecular weight(s) of the protein(s) of interest; i.e., the smaller the molecular weight of the protein, the higher the percentage of mono- or bisacrylamide, and conversely, the larger the molecular weight of the protein, the lower the percentage of mono- or bisacrylamide.

Uniform-concentration gels have separation ranges as shown in *Table 2*, and gradient gels have a separation range as shown in *Table 3*. Gels can be purchased ready-made or can be produced in the laboratory according to procedures in ⟨1056⟩.

Table 2. Linear Range of Separation (kD) for Uniform-Concentration Gels

Acrylamide Concentration (%)	Linear Range of Separation (kD)
5	57–212
7.5	36–94
10	20–80
12	12–60
15	10–43

Table 3. Linear Range of Separation (kD) for Gradient Gels

Acrylamide (%)	Protein Range (kD)
5–15	20–250
5–20	10–200
10–20	10–150
8–20	8–150

SAMPLES AND STANDARD

To prepare samples, analysts typically must lyse cells and tissues in order to release the proteins of interest. The main consideration when choosing a lysis buffer is whether the antibody chosen for detection of the protein(s) of interest can recognize denatured samples. When this is not the case, analysts use buffers without detergent or with relatively mild, nonionic detergent.

Samples should be treated (e.g., reduced, nonreduced, or denatured) according to general chapter ⟨1056⟩, and when a sample of unknown protein content is used, a series of dilutions should be loaded onto the gel. Standards (molecular weight markers) should be treated according to the manufacturer's instructions.

ELECTROPHORESIS

Before applying samples to the stacking gel wells according to ⟨1056⟩, analysts denature samples (e.g., heat at 95°–100° for 5 min). An appropriate volume of sample is loaded onto the gel, and a voltage of 8 V/cm applied until the dye has moved into the resolving gel. Afterward, the voltage is increased to 15 V/cm, and the separation is run until the bromophenol blue reaches the bottom. If a commercially available gel is used, the manufacturer's recommendations are followed. *Table 4* shows common sample-loading volumes for particular gels.

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Table 4. Common Sample-Loading Volumes

Wells	Gel Thickness (mm)	Maximum Sample Load Volume (µL)
10	1.0	25
10	1.5	37
12	1.0	20
15	1.0	15
15	1.5	25

TRANSFER

After electrophoresis, the proteins of interest can be blotted to a membrane such as nitrocellulose or PVDF with a pore size that is appropriate for the molecular weight of the proteins of interest. Both nitrocellulose and PVDF have a protein-binding capacity of about 100–200 µg/cm². PVDF is more chemically resistant than nitrocellulose and is easier to handle. Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus and vary depending on the system.

Transfer can be done in wet or semi-dry conditions. Semi-dry transfer generally is faster, but wet transfer is especially recommended for large proteins >100 kD. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

A standard buffer for transfer is the same as the buffer used for the migration or running buffer without SDS, but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kD, SDS should be included at a final concentration of 0.1%. Lowering methanol in the transfer buffer also promotes swelling of the gel, allowing large proteins to transfer more easily. *Table 5* contains common buffers used for Western blot methods.

Table 5. Common Western Blot Buffer Formulations

Buffer	Content
Sample buffer 2× (nonreducing) 1D electrophoresis	1.89 g of Tris 5.0 g of SDS 50 mg of bromophenol blue 25.0 mL of glycerol 100 mL of water Adjust with HCl to a pH of 6.8. Add water to 125 mL.
Sample buffer 2× (reducing) 1D electrophoresis	To nonreducing sample buffer: Add 12.5 mL of 2-mercaptoethanol before adjusting the pH. Alternatively, use 1.93 g of Tris, and add a suitable quantity of DTT[a] to obtain a final concentration of 100 mM DTT.
Running buffer 10× 1D electrophoresis	151.4 g of Tris 721.0 g of glycine 50.0 g of SDS Add water to 5000 mL. Adjust to a pH of 8.1–8.8.
Transfer buffer 10×	151.4 g of Tris 721.0 g of glycine Add water to 5000 mL. Adjust to a pH of 8.1–8.8.
Transfer buffer 1×	100 mL of 10× stock 500 mL of water 200 mL of methanol Add water to 1000 mL.
TBS 10×	24.23 g of Tris base 80.06 g of NaCl Mix in 800 mL of ultrapure water. Adjust with pure HCl to a pH of 7.6. Add water to 1000 mL.
TBS-T	100 mL of TBS 10× 900 mL of water 1 mL of polysorbate 20
8.5 M urea stock	510 g of urea Add water to 1000 mL.

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Table 5. Common Western Blot Buffer Formulations *(continued)*

Buffer	Content
Sample buffer 2D electrophoresis	47 mL of 8.5 M urea stock 385 mg of tributyl phosphine (TBP) 2 g of CHAPS[b] 25 mg of bromophenol blue 1% carrier ampholytes of choice

[a] Dithiothreitol.
[b] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Methanol is necessary only if analysts use nitrocellulose. If they use PVDF, they can remove methanol from the transfer buffer and need only to activate the PVDF before they assemble the gel and membrane sandwich.

In semidry transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between the cathode and anode. During wet transfer the membrane should be closest to the positive electrode, and the gel should be closest to the negative electrode. The composition of the transfer buffer is not necessarily the same as the migration or running buffer. Analysts should consult the apparatus manufacturer's protocol, and it is common to add both SDS and methanol. The balance of SDS and methanol in the transfer buffer, the proteins' molecular weights, and the gel percentage can affect transfer efficiency for both wet and semidry transfers.

BLOCKING

Blocking the membrane prevents nonspecific background binding of the primary and secondary antibodies to the membrane. Traditionally, one of two blocking solutions is used: nonfat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phosphoproteins. To prepare a 5% milk or BSA solution, weigh 5 g/100 mL of Tris-buffered saline containing polysorbate 20 buffer (TBS-T; see *Table 5*). Mix well, and filter. Failure to filter can lead to spotting in which tiny dark grains contaminate the blot during development. Incubate at 4° for 1 h with gentle shaking. Rinse in TBS-T after the incubation.

PRIMARY ANTIBODY AND INCUBATION BUFFER

Dilute the antibody with blocking buffer at a proper dilution (1:100–1:3000, depending on antibody titer), and optimize the dilution according to the results. Too much antibody can result in nonspecific bands.

INCUBATION TIME

Incubation time can vary between a few hours and overnight, and depends on the binding affinity of the antibody for the protein and the abundance of protein. A more dilute antibody with a prolonged incubation may improve specific binding.

INCUBATION TEMPERATURE

It is best to incubate under cold temperatures. When analysts incubate in blocking buffer overnight, they should incubate at 4° to prevent contamination from bacterial growth, and should gently agitate the antibody solution to enable adequate homogeneous covering of the membrane.

SECONDARY ANTIBODY AND INCUBATION BUFFER

Handle the secondary antibody and incubation buffer as follows. Wash the membrane several times in TBS-T while agitating to remove residual primary antibody. Dilute the secondary antibody with TBS-T at the suggested dilution. Too much secondary antibody can result in nonspecific bands. Incubate the blot at room temperature for 1–2 h with gentle agitation. *Table 1* shows multiple options for secondary detection reagents and methods. More details are available in the *Immunoblot Data Analysis* section below.

Slot/Dot Blot

The procedure is similar to the procedure for 1D immunoblotting, but differs because protein samples are not separated electrophoretically but are spotted directly onto the membrane either manually or by use of a blotting unit (dot or slot blot format).

PROCEDURE USING MANUAL SPOTTING

Handle manual spotting as follows. Place a dry filter paper on a stack of dry paper towels. Place filter paper that is pre-wet with transfer buffer on top of the dry filter paper. Place a pre-wet membrane on top of the pre-wet filter paper. Samples are spotted onto the pre-wet membrane and are allowed to absorb into the membrane. After the sample is absorbed, place the membrane on a clean, dry filter paper to dry.

PROCEDURE USING A VACUUM-BLOTTING UNIT

Analysts typically use a vacuum-blotting unit as follows. Prepare a membrane, and place it in the blotting unit according to the manufacturer's instructions. Apply vacuum to the blotting unit to remove excess buffer. To improve solubility, dissolve the

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sample in a buffer, and if it is not clear, remove precipitates by centrifugation. If the sample is too viscous to pipet, then dilute it further with buffer. With the vacuum off, carefully pipet samples into the wells, and apply vacuum to the blotting unit. After all the samples have filtered through the membrane, turn off the vacuum, add buffer to each well to wash down the sides, and apply vacuum again. Remove the membrane, and proceed with immunoblotting.

2D Immunoblotting

SAMPLE PREPARATION

The compounds used to solubilize proteins must not increase the ionic strength of the solution. For example, a common sample solubilization solution is the following: 8 M urea, 50 mM dithiothreitol (DTT) or 2 mM tributyl phosphine (TBP), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, and 0.0002% bromophenol blue. The addition of carrier ampholytes enhances the solubility of proteins as they approach their isoelectric points. The use of ampholytes produces an approximately uniform conductivity across the pH gradient without affecting its shape, meaning that the concentration of carrier ampholytes should be optimized.

CHARGE SEPARATION

Several vendors produce and sell immobilized pH gradient (IPG) strips, or they can be made in-house according to ⟨1054⟩. The choice of IPG strips depends on the pI of the proteins of interest. The size of the IPG should match the size of the second-dimension gel. The amount of protein in each sample should be determined, and the amounts loaded on the IPG strips should be in the range of 10–300 µg, depending on the size of the IPG. The sample and standard should be loaded according to the manufacturer's instructions or according to ⟨1054⟩. Analysts then proceed with the isoelectric focusing by applying the electrical parameters described in ⟨1054⟩ or by the manufacturer.

MOLECULAR WEIGHT SEPARATION

After charge separation, analysts must equilibrate the strip in SDS-containing buffer before separation in the second dimension to determine molecular weight (as described previously in the *1D Immunoblotting* section). Analysts should position the strip directly on top of the gel, then secure the strip by overlaying it with 0.5%–1.0% agarose prepared in SDS–PAGE running buffer. To track the ion front in the second dimension, analysts can add bromophenol blue to the agarose.

IMMUNOBLOT DATA ANALYSIS

The presence or absence of bands usually is determined by comparison to a control (highly characterized antigens known or qualified to give a precise or expected response) of a type that is similar to the antigen being processed. Although analysts usually perform a qualitative comparison, bands or dots can be quantitated using a detection system (e.g., after incubating in a solution containing 4-CN peroxidase substrate; also see *Table 1*), and are compared to the control bands run in parallel (e.g., in the same gel).

Detection Options

ENHANCED CHEMILUMINESCENCE

Enhanced chemiluminescence is a popular method for detection in immunoblot analysis because it is highly sensitive (detection to pg or lower levels), and can be used to quantitate the relative concentration of the protein of interest. The method depends on incubation of the blot with a substrate that luminesces when exposed to the reporter on the secondary antibody. The light is detected using either photographic film or a charge-coupled device (CCD) camera. The image then is analyzed by densitometry to evaluate the relative amount of protein staining in terms of optical density. By using an appropriate set of molecular weight standards as markers, analysts can estimate molecular weight.

FLUORESCENCE DETECTION

Direct fluorescence can be used to detect proteins on blots. Direct fluorescence is simple, rapid, sensitive, and has a greater linear range than enhanced chemiluminescent detection. The advantage of direct fluorescence is the ability to detect many different fluorescent signals. This analysis avoids the need to reprobe the blot. Compared to enhanced chemiluminescence, fluorescence methods are easier to visualize and quantitate on CCD or laser-scanning imaging systems. Some data-acquisition systems permit extending the time of data acquisition to optimize signal-to-noise levels. Fluorescence-labeled blots that can be re-examined are useful for this purpose.

Enhanced chemifluorescence (ECF) is another common fluorescence method. ECF uses secondary antibodies conjugated to either HRP or AP. The enzyme-conjugated antibodies react with specific substrates that produce fluorescence after enzymatic cleavage. Analysts visualize the resulting signals using UV epi-illumination and capture digital images. An ECF signal has a greater linear range than traditional enhanced chemiluminescence. For example, direct fluorescence has a limit of detection in the pg range, and also has about 2 logs of linear dynamic range.

Quantum dots also are an alternative to detect proteins in immunoblot analysis. Quantum dots are a type of probe that can be conjugated to antibodies simultaneously or sequentially to detect multiply labeled antigens, without the need for blot stripping. Similarly, near-infrared (NIR) fluorophore–linked antibody is a method for antibody detection whereby light produced

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from the excitation of a fluorescent dye is measured in a static state. Light measured in a static state allows more precise and accurate detection than light measured in a dynamic state (e.g., chemiluminescence).

RADIOACTIVE DETECTION

Proteins also can be detected by labeling an antigen with a radioactive isotope (e.g., iodine). On the one hand, this method has the advantage that the radioactivity in a band is easy to quantitate by means of time exposure to film and densitometry, or by directly excising the band from the membrane and counting using a scintillation counter. On the other hand, radioactivity also introduces the disadvantage of safety because analysts must manage radioactive material, and analytical laboratories must have a program to control and monitor waste management and individual exposure.

Immunoblot Quantitation

NONELECTROPHORETIC QUANTITATION

The quantitation of a specific protein is achieved when the blot procedure is properly optimized and generates a linear response range over a particular time frame. Immunoblot quantitation includes several elements: adequate antigen and antibody concentrations and purity, antibody specificity, blocking conditions, sufficient washes, and the duration and intensity of the signals. Once the exposures are captured on a film or electronically under optimized conditions, analysts use densitometric methods to quantitate results by comparing a specific protein on the blot and on the standard. Analysts can correct results for background by including a negative control.

The intensity of the bands depends on the amount of protein. Different commercial software packages are available for image analysis of bands on a film. Alternatively, digital imaging systems containing CCD cameras usually include software designed to perform data analysis.

ELECTROPHORETIC QUANTITATION

Proteins of various molecular weights are identified by the extrapolation of plots of relative mobilities of prestained proteins of known molecular weight and can be compared to the positive control. Positive controls are trended to determine the limit range of the densitometry results compared to the nominal concentration results. Independent of the detection method, the following criteria must be met for a valid Western blot result.

- Ensure adequate development by minimizing membrane overexposure and visualizing staining controls.
- The prestained molecular weight markers must be visible and must cover the anticipated range.
- The band(s) should have the appropriate location and intensity for the standard, the control, and the protein of interest.
- There should be no blot or staining artifacts that obscure the visualization and interpretation of bands.

METHOD VALIDATION

As outlined by ICH Guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology (effective November 2005) and *USP* general chapter *Validation of Compendial Procedures* ⟨1225⟩, a qualitative assay such as the slot/dot blot requires validation of specificity. Specificity is the ability to detect the analyte in the presence of other components. For validation, it should be shown that the particular steps of the slot/dot blot method can detect the antigen when present and do not report false positive results when the antigen is absent. In addition, demonstration of the specificity of the antigen-specific antibodies is part of the specificity evaluation.

USP general chapter ⟨1225⟩ provides guidelines for the validation of analytical procedures, and analysts should consider this resource when they validate immunoblot methods. All methods require a demonstration of the specificity of the antibody to the antigen and the lack of recognition of other proteins and reagents in the matrix. Identity tests require only specificity. Limit tests require specificity and LOD. A sensitivity control incorporated into each test can show that the LOQ is met on each determination to account for potential changes in the sensitivity of the method. A quantitative test requires all ICH validation parameters, including robustness testing.

Demonstration of electrophoretic immunoblot specificity should include the following: stained gels to show protein separation, stained blots to show adequate protein transfer to the membrane, blots with control samples to show the specificity of the conjugate to the primary antibody, and blots that show the binding of the antibody to the appropriate antigen. Method validation also can identify the need for control membranes for each assay, as well as protein sensitivity controls as measures of system suitability.

⟨1105⟩ IMMUNOLOGICAL TEST METHODS—SURFACE PLASMON RESONANCE

Introduction

Surface plasmon resonance (SPR) optical detection is a useful method for the label-free assays (procedures) that study biomolecular interactions. Commercially available SPR biosensors that incorporate these assays can collect real-time, information-rich data from binding events. These data can be used widely from basic research to drug discovery and development to manufacturing and quality control (QC). SPR can characterize binding events with samples ranging from

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proteins, nucleic acids, and small molecules to complex mixtures, lipid vesicles, viruses, bacteria, and eukaryotic cells. Typical quality and safety attributes addressed with SPR analysis include:

- Interaction specificity
- Interaction affinity
- Kinetic binding parameters
- Thermodynamic parameters
- Biologically active concentration of an analyte

This chapter provides an overview of the physics underlying SPR and common instrument configurations, as well as the range of molecules that can be studied and general considerations for experimental design as determined by the assay objective.

Overview

History

The physical principles of SPR were first explained in the early 1900s, starting with a description of the uneven distribution of light in a diffraction grating spectrum caused by the excitation of surface plasmon waves. A landmark series of experiments showed the optical excitation of surface plasmons under conditions of total internal reflection and fostered detailed studies of the application of SPR for chemical and biological sensing. Since then, SPR's potential for characterizing thin films and monitoring interactions at metal interfaces has been recognized, and significant research and development have yielded instruments that can quantitatively evaluate the binding interactions of small and large molecules.

Physics

SPR is an optical phenomenon that occurs when a thin conducting film is placed between two media that have different refractive indices. In many commercially available instruments, the two media are glass and the sample solution, and the conducting film is preferentially a gold layer applied to the glass, although other conducting metals such as silver have been used. The glass–metal component comprises a solid support that is often referred to as a *sensor*.

Light applied to the glass under conditions of total internal reflection produces an electromagnetic component that is called an *evanescent wave*. The evanescent wave penetrates the medium of lower refractive index (typically the sample solution) without losing net energy. The amplitude of the evanescent wave decays exponentially with distance from the surface, roughly one-half of the wavelength of the incident light (e.g., for a light source of 760 nm the evanescent wave penetrates approximately 300 nm).

For a specific combination of wavelength and angle of incident light, electron charge density waves called *plasmons* are excited in the gold film. As energy is absorbed via the evanescent wave, a decrease in the intensity of the reflected light at a specific angle (the SPR angle) is observed. Analysts can conduct an SPR experiment by fixing the wavelength and varying the angle of incident light.

An increase in mass at the sensor surface caused by a binding interaction between two or more molecules causes a change in the local refractive index (RI) that gives rise to an SPR response, which is observed as a shift in the SPR angle. By monitoring the shift in the SPR angle as a function of time, an analyst can generate a *sensorgram* (*Figure 1*). The change in RI is very similar for different proteins, so the SPR measurement depends primarily on the mass change at the sensor surface and is relatively independent of the nature of the molecules being measured.

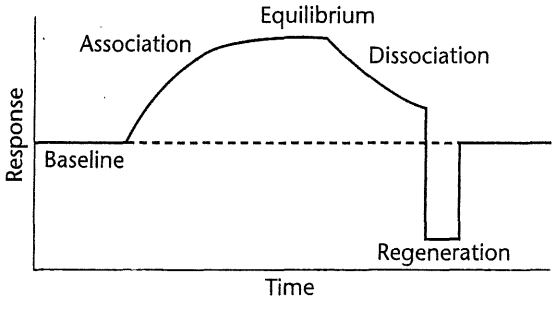

Figure 1. Representative sensorgram.

Instruments

The main components of commercially available SPR instruments are (1) a light source, typically a high-efficiency light-emitting diode, (2) an optical detector such as a diode-array or charge-coupled device camera, (3) a solid support containing the conducting film and some means for attaching molecules, (4) a sample delivery system, frequently a microfluidic device capable of delivering samples using single serial or parallel injections via single or multiple needles, and (5) a computer with appropriate software for instrument control, data collection, and analysis.

Prism-based and diffraction-grating instrument systems are commercially available. Most prism-based systems follow the Kretschmann configuration (*Figure 2*). The light is focused onto the sensor surface (away from the samples) via a prism with a refractive index matching that of the surface. In this configuration the incident light does not penetrate the sample solution, which permits SPR measurements for heterogeneous, turbid, or opaque samples. In systems that utilize a diffraction grating (*Figure 3*) the analyte solution is placed over a plastic surface on which a metal has been deposited. The plastic acts as an attenuated total internal reflection prism in which light reflected from the grating is reflected many times back to the grating surface. In this configuration light passes through the analyte sample solution, and thus turbid or opaque samples are not suitable for measurement. The diffraction grating does permit sampling of a larger surface area and is applicable for SPR measurements of arrays.

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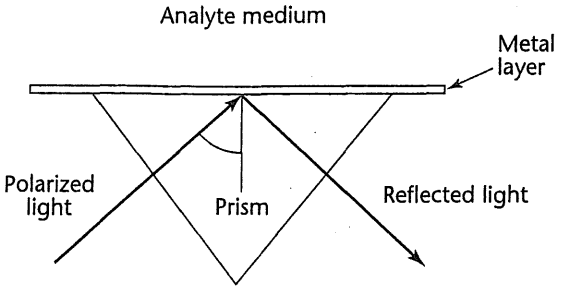

Figure 2. Kretschmann SPR configuration.

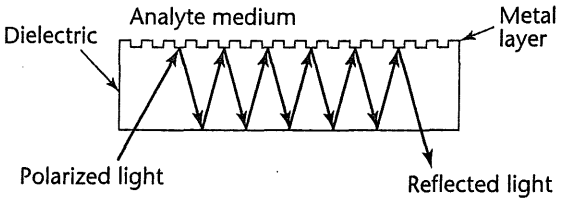

Figure 3. Diffraction grating SPR configuration.

The instruments are compatible with a wide range of biological samples and buffers as well as some organic solvents.

Biomolecular Interactions That Can Be Studied By Assays Using SPR

A diverse range of biological entities can be studied using SPR, including small molecules (<100 Da), proteins, nucleic acids, lipids, bacteria, viruses, and whole cells. Most published SPR research involves protein–protein interactions, of which antibody–antigen interactions represent a dominant subset. Improvements in instrument sensitivity and experimental protocols have helped analysts make studies of small molecules, lipids, and nucleic acids. Protein interactions with larger entities such as whole cells and some bacteria and viruses are limited by the exponential decay of the evanescent wave as described above. In practice these large molecules can be studied effectively, but the information obtained may be limited to qualitative or semiquantitative (e.g., relative ranking) data.

Assay Types

Several types of SPR assays are useful, including binding specificity, concentration analysis, kinetics and affinity analysis, and thermodynamics. Each assay type generates unique information that is helpful for profiling biomolecules.

SPR is also suitable for use in qualitative studies to confirm the specificity of interactions. Analysts can monitor a number of sequential binding events because each individual event yields a mass increase on the sensor chip surface, and all stages in the binding process are monitored. Examples include epitope mapping, antibody isotyping, and immunogenicity measurements.

Most chemical and spectroscopic methods used to quantify proteins (1) measure total protein content, (2) do not distinguish active from inactive molecules, and (3) cannot be used in conjunction with unpurified samples. Because SPR is a noninvasive method (no light penetrates the sample), it can measure small amounts of analyte molecules from complex matrices such as food products, serum or plasma, and cell extracts. Direct or indirect (inhibition or competitive) formats for measuring concentration are possible. SPR biosensors are uniquely suited for measurement of kinetic association and dissociation rate constants from real-time measurement of binding interactions. Affinity can be derived either from interactions that have reached equilibrium or from the ratio of the dissociation and association rate constants. The typical working range for affinity measurements is pM to high μM concentrations. Association rate constants that can be measured typically range from 10^3 to 10^7 $M^{-1}s^{-1}$ and dissociation rate constants from 10^{-5} to 0.5 s^{-1}. By studying temperature dependence of rate and affinity constants, analysts can determine thermodynamic parameters for a binding interaction. Not only can the equilibrium values for changes in enthalpy (ΔH) and entropy (ΔS) associated with complex formation be determined, but transition state energetics can also be evaluated. Subsequent sections of this chapter address the specific details for these different assay types.

The SPR Assay

The typical SPR assay involves five steps:

1. Sample and buffer preparation
2. Surface preparation
3. Analyte binding
4. Surface regeneration
5. Data analysis and interpretation

Careful attention to experimental design leads to high-quality data and results. In SPR experiments, mass transport is essential for binding interactions to take place in instruments that use thin-layer flow-cell systems. Analyte molecules

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are transferred from the bulk solution to the binding surface via mass transport. When a limitation for binding occurs as a result of fast binding kinetics combined with high surface density, the binding interaction is considered mass-transport limited. In this case, the binding kinetics and complex formation are influenced by the availability of analyte molecules. The advantages and disadvantages of mass-transport–limited binding are discussed later in the application examples.

Sample and Buffer Preparation

Both purified and crude samples can be analyzed in a variety of matrices including serum, plasma, cell supernatants, and lysates. Crude samples containing particulates (e.g., cell debris or precipitates) may require clarification in order to help minimize unwanted binding. A short spin (30–60 s) in a benchtop centrifuge or filtration (0.22–1.0 µm) using low–protein-binding filters is recommended. The concentration range for evaluation depends on the experimental objective (yes/no binding, concentration, or kinetic/affinity analysis) as well as the binding affinity of the interacting molecules. In general, sample concentrations an order of magnitude below the equilibrium dissociation constant (K_D), can be detected by SPR, but determination of an exact concentration is influenced by the analyte size (large vs. small molecules), binding specificity, and overall biological activity of the samples.

Most biological buffers and several organic solvents can be used in SPR experiments. The addition of salts and detergents to buffer solutions frequently can stabilize biomolecules. High-quality grade (e.g., molecular biology grade or higher) buffer components should be used. To simplify experiments, analysts should add only components that are absolutely required for biological activity or function. Buffers should be filtered and degassed before use.

Surface Preparation

Surface preparation involves the attachment of one of the binding partners to a solid support (surface). This process is frequently referred to as *immobilization*, and the resulting surface with the attached biomolecule is the sensor for the experiment. The choices of binding partner, solid support, and immobilization method are influenced by (1) the nature and demands of the application or experimental objective; (2) the availability of surfaces with different properties (e.g., charge density, hydrophobicity, or hydrophilicity); (3) the characteristics and supply of biomolecule to be used for immobilization; and, most importantly, that (4) biological activity be maintained and binding sites be available to interacting partners. Depending on the experimental objective, homogeneous or orientation-specific attachment of biomolecules also may be desired. The two main categories of immobilization methods are (1) direct immobilization, in which the molecule is covalently attached to the surface, and (2) indirect or capture immobilization, which takes advantage of tags or native groups on the protein or biomolecules (*Table 1*).

Table 1. Surface Preparation Techniques

Chemistry	Immobilization Method	Biomolecules	Comments
Amine	Direct	Proteins, peptides	Amino terminus, Lys residues
Thiol—native	Direct	Proteins, peptides	Native Cys residue
Thiol—added	Direct	Proteins, peptides	Carboxyl groups derivatized
Aldehyde	Direct	Glycoproteins	*Cis–diol* required
Biotin capture	Indirect	Biotinylated peptides, nucleic acids, proteins	Stable, irreversible capture
Affinity tags	Indirect	Proteins, peptides	His, Glutathione *S*-transferase (GST), etc.
Protein A, Protein G	Indirect	Antibodies, IgG-tagged molecules	IgG species–dependent
Protein A, Protein G	Indirect	Biomolecules specific to the capturing antibody	Mono- or polyclonal antibodies may be suitable—testing recommended
Hydrophobic adsorption, membrane capture	Indirect	Lipids, membranes, membrane-associated proteins	Monolayer or bilayer attachment possible

Direct Immobilization: For direct immobilization, several chemistries are available for attaching proteins or other biomolecules to the surface. The properties of the surface determine the specific sequence of steps and length of time required to prepare the surface. Many commercially available surfaces have a biologically compatible layer (e.g., a hydrogel) that contains functional groups such as carboxyl that can be used for immobilization. To ensure binding specificity, the purity of the biomolecule that is attached to the surface should be 95% or greater and the required concentration should range from 1 to 1000 µg/mL. Direct immobilization chemistries frequently result in heterogeneous surfaces because of random orientation of biomolecules on the surface.

Immobilization via free primary amine groups such as lysine residues in proteins or the amino terminus of proteins or peptides is one of the most generally applicable covalent chemistries for attaching proteins to a surface. Carboxyl groups on the surface are converted to reactive esters using a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) or sulfo-NHS (sNHS). The protein or biomolecule is applied in high concentrations (mg/mL) to maximize the efficiency of amine coupling. Finally, free esters are blocked with ethanolamine. The contact time with the surface, the protein concentration, or the EDC/NHS concentration can be varied to adjust the immobilization level.

When amine coupling interferes with the binding site, the biomolecule can be attached using alternative coupling chemistries or a high-affinity capture approach. For example, for biomolecules with free thiol groups (typically cysteine residues), a disulfide group is introduced by treating the surface with NHS and EDC to attach 2-(2-pyridinyldithio)ethaneamine (PDEA). Adding the biomolecule to the surface results in thiol–disulfide exchange, and excess PDEA groups are inactivated with cysteine–HCl. If the biomolecule lacks a free thiol group, a reactive disulfide (PDEA) can be linked to carboxyl groups. Subsequently the pyridyldisulfide groups can be

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attached to thiol groups on the surface that have been derivatized via injection of NHS and EDC, followed by cystamine, then reduction with dithioerythritol (DTE) or dithiothreitol (DTT). Attachment of maleimide groups to the surface makes possible an alternative form of immobilization via thiol groups in which a stable thioether bond is formed. Surfaces prepared using this method have the capacity to withstand basic pH (> 9.5) and reducing agents such as β-mercaptoethanol and dithiothreitol. Several heterobifunctional reagents are available commercially for introduction of reactive maleimido groups to the surface, including sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester), sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate), GMBS [*N*-(γ-maleimidobutyrloxy)sulfosuccinimide ester], EMCH [*N*-(ε-maleimidocaproic acid)-hydrazide] or BMPH [*N*-(β-maleimidopropionic acid)-hydrazide]. For biomolecules containing either native aldhehyde groups or *cis–diols*, which may be converted into aldehydes by mild oxidation, surface attachment via a hydrazone bond is an option. Hydrazide groups on the sensor surface react with aldehyde groups on the biomolecule to form a stable bond. Immobilization via aldehyde groups is most useful for glycoconjugates, glycoproteins, and polysaccharides.

Indirect (High-affinity Capture) Immobilization: Indirect or high-affinity capture immobilization approaches use tags commonly used for protein purification. This technique exploits the high-affinity capture of the biomolecule by a capturing molecule that has been immobilized covalently using one of the techniques described above. The requirement for biomolecular purity is less stringent for indirect versus direct immobilization because the capturing step for the biomolecule can also provide purification. Indirect immobilization frequently yields a homogenous surface because all biomolecules are oriented similarly via the tag. The affinity between the biomolecule and its capturing agent should be sufficiently high to ensure little or no dissociation from the surface for the duration of an analysis cycle. Monoclonal antibodies are frequently used as capture molecules. For example anti-GST antibodies can be attached to the sensor chip surface via amine chemistry in order to capture GST-tagged molecules. Protein A, Protein G, and anti-IgG antibodies are useful capturing molecules for use with antibodies.

The high-affinity interaction between streptavidin or related molecules and biotin ($K_D \approx 10^{-15}$ M) makes it a useful system for the capture of biotinylated molecules (e.g., proteins, peptides, nucleic acids, membranes, and liposomes). Frequently, the biotin-binding protein is attached to the surface using primary amine groups. Because of the high affinity of the interaction, biotinylated molecules are considered permanently immobilized, and in contrast to most other capture approaches biotinylated molecules cannot be removed without damaging the surface. Histidine (His)-tagged recombinant proteins can be captured via nickel–NTA chemistry or covalently immobilized anti-His antibodies.

Lipids and membrane-associated proteins can be captured to the surface as either a lipid monolayer or bilayer. Lipids from micelles or liposomes adsorb to a hydrophobic surface, creating a lipid monolayer with the hydrophobic lipid tails oriented toward the solid support and the hydrophilic heads towards the aqueous sample. This approach provides a stable environment for proteins associated with a membrane surface or partially inserted into the membrane, but it is not ideal for transmembrane proteins because the resulting surface presents only half the membrane structure for binding interactions. Intact membrane structures (lipid bilayers) with associated or incorporated proteins can be captured by preparing liposomes with a specific antigenic component or with biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin, respectively.

Additional Considerations: Once the biomolecule has been attached to the sensor using either a direct or indirect immobilization approach, analysts should assess the baseline stability of the newly created surface. If the baseline is decreasing (downward drift), the most likely cause is the presence of unattached biomolecules, possibly because of self-association or aggregation. If the baseline is increasing (upward drift) refolding or re-orientation may be causing the change. In either case, the newly created surface should be conditioned before use by one or more of the following: (1) multiple injections of biologically compatible buffer; (2) washing the surface with buffer at a fast flow rate; (3) multiple injections of either high ionic strength (e.g., 1 M NaCl) or detergent (e.g., 20 mM CHAPS or 0.05% Polysorbate 20 (P20)) solutions; or (4) repeated analyte binding and regeneration injections. NOTE: recommendations (3) and (4) should be used only if the activity of the biomolecule in the presence of these reagents has been evaluated previously.

Large baseline drifts caused by low-affinity capture may be overcome by using EDC/NHS as a cross-linking step, but this may compromise biomolecule activity if active sites of the biomolecule are involved in the cross-linkages. The effect of cross-linking on biomolecule activity must be tested empirically for each biomolecule–analyte system. In general, cross-linking should be as brief as possible: 15 s is often sufficient to achieve acceptable baseline stability without compromising biomolecule activity.

How Much to Immobilize: The amount of biomolecule to immobilize depends on the experimental objective. Equations 1 and 2 are useful for calculating the appropriate surface density:

$$R_{max} = (MW_A/MW_L) \times R_L \times S_m \quad \text{[Equation 1]}$$

$$R_L = R_{max} \times (1/S_m) \times (MW_L/MW_A) \quad \text{[Equation 2]}$$

R_{max} = theoretical maximum binding response (assuming a surface that is 100% active and 100% bound with analyte)
R_L = response of the immobilized molecule
MW_A = molecular weight of the analyte
MW_L = molecular weight of the immobilized molecule
S_m = molar binding stoichiometry

For kinetic experiments, a low density of immobilized molecule is preferred in order to avoid steric hindrance, aggregation, and/or mass-transport–limited binding. Low density is defined as R_L that limits R_{max} to 5–50 response units. For other applications, e.g., concentration analysis where mass-transport–limited binding is desired, R_{max}

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can be 100–200 times higher than for kinetic experiments provided that steric hindrance or aggregation are not induced. Specific recommendations for immobilization density are included in the application examples for this chapter.

Analyte Binding

Samples that will be evaluated for binding using SPR do not require the same purity as biomolecules intended for direct immobilization onto the surface. Because the light source does not penetrate the sample, turbid or opaque samples can be analyzed by SPR. Whenever practical, samples should be clarified according to the recommendations given under *Sample and Buffer Preparation*, and buffer additives should be minimized, including only the amount required for biological activity.

Differences between the refractive index of the bulk and sample buffers give rise to a response. The use of control surfaces and samples aids in demonstrating binding specificity for the molecules in SPR. For direct immobilization methods, suitable control surfaces can be (1) the sensor surface without any modification or biomolecule attached, (2) a surface that has been chemically treated in the same manner as the surface containing the biomolecule, or (3) a related but known nonbinding biomolecule. For surfaces prepared using indirect (capture) immobilization the capturing molecule in the absence of the tagged binding partner should be used as the control surface. The difference in response between the control and active surfaces gives an initial indication of the binding specificity. Concentration-dependent responses and inhibition of binding by incubating the sample with the biomolecule on the surface can further establish the binding specificity.

If nonspecific or unwanted binding is observed, analysts should determine the source. Frequently changes in pH or ionic strength of the buffers used in the experiment will reduce or eliminate the unwanted binding. Additional suggestions for reducing nonspecific binding are summarized in *Table 2*.

Table 2. Suggested Actions for Reducing Nonspecific Binding

Category	Action
Experimental Design	1. Optimize running buffers: A. increase salt (150 to 500 mM) B. add detergent (0.001% to 0.05%) C. match composition of sample and running buffers 2. Change ligand immobilization method 3. Evaluate ligand quality 4. Increase or decrease temperature in detection chamber
Choice of Surface	1. Change properties of sensor surface: A. reduce electrostatic interactions B. evaluate hydrophobic vs. hydrophilic character of surface C. consider alternative ligand to use for control surface 2. Pre-immobilize amino—PEG 3. Change blocking molecule (e.g., ethylenediamine)
Additions to Sample	1. Add nonspecific binding reducer to sample: A. increase ionic strength of running and sample buffers (e.g. 150 to 500 mM NaCl) B. add detergent to running and sample buffers (e.g. 0.001% to 0.05% surfactant P20) C. add soluble carboxymethyl dextran (1–10 mg/mL, for dextran-based surfaces only) 2. Simplify sample buffer—include only components required for biological activity 3. Evaluate analyte quality

Equations 1 and 2 are also useful for assessing surface activity. The higher the binding response, the more active the surface is unless the observed binding response exceeds the calculated R_{max} value. In this case, the molar binding stoichiometry is incorrect, the analyte molecule is aggregated, or the analyte is binding nonspecifically to the surface. Binding responses that are low (< 10% of R_{max}) suggest that the analyte concentration selected for the experiment is too low or that the surface activity of the immobilized molecule is low. In the former case, increasing the analyte concentration should increase the binding response, and in the latter situation using a different immobilization method may be helpful.

Surface Regeneration

Surface regeneration refers to the process of removing bound analyte from the surface in order to reuse the surface for subsequent binding interactions. In some instances, complex dissociation is fast and bound analyte is simply washed away with buffer, so regeneration is not needed. Alternatively, the instrument configuration may allow multiple samples to be injected either sequentially or in parallel across several immobilized surfaces simultaneously, thereby limiting the need for regeneration. Inadequate surface regeneration may affect the reproducibility of an assay and negatively affect the overall quality of the resulting data. To identify the correct conditions, analysts should consider the nature of the specific interaction and the experimental objective. For example, a slight baseline drift will not affect the results when a simple yes/no answer is sought, but in concentration determination or kinetic studies, optimization of the regeneration step is critical.

Most biochemical interactions involve non-covalent bonds such as hydrogen, electrostatic, van der Waals, and hydrophobic bonds. Because the combination of physical forces responsible for binding and the regeneration conditions critical for not causing irreversible conformational changes are unknown for most interactions, the final conditions must be evaluated empirically.

The ideal condition for regeneration dissociates all the bound material without affecting the biological properties of the immobilized biomolecule. An incomplete regeneration or too stringent conditions may result in decreased analyte binding capacity in subsequent cycles because of either blocking of binding sites by nondissociated analyte

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or partial denaturation of the biomolecule. Regeneration buffers and solutions can be divided into different classes by the effect they have on the interaction. Any combination of buffers can be used.

The major classes of regeneration buffers are: acidic, basic, ionic/chaotropic, detergent, hydrophobic/nonpolar, and chelating (see *Table 3* for examples of each class). Analysts should start with mild conditions, moving progressively to more harsh conditions. In many cases, especially when one is working with antibodies, change in pH is the most effective method of regenerating the surface. Contact time with the surface is important for efficient regeneration. When analysts use pH change, the contact times should be short, one-half to 2 min. When analysts use high ionic strength or chaotropes, longer contact times of 2–4 min are usually effective.

Table 3. Examples of Regeneration Solutions

Acid	Base	Ionic/ Chaotropic	Detergent	Hydrophobic/ Nonpolar	Chelating
1–100 mM HCl	1–100 mM NaOH	0.5–5 M NaCl	0.02%–0.5% SDS	25%–100% ethylene glycol	10–20 mM EDTA or EGTA
10–100 mM glycine, pH 1.3–3.0	10–100 mM glycine, pH 9.0–10.0	1–4 M $MgCl_2$	40 mM octyleneglycol + 20 mM CHAPS	5%–50% DMSO	10–200 mM imidazole
10–100 mM phosphoric acid	1 M ethanolamine HCl, pH 9.0 or above	1 M KSCN	40 mM octylglucoside, 40 mM octylglucoside	1%–10% acetonitrile	
0.1% TFA	100 mM sodium carbonate + 1 M NaCl, pH 9–11	2–6 M guanidine HCl			
100 mM Formic acid	20–100 mM NaOH containing 0.5% surfactant P20 or 0.05% SDS				

The purpose of optimizing the regeneration conditions is to find the mildest possible regeneration solution that completely dissociates the complex. Analysts should maintain a constant level of activity over the binding–regeneration cycles even if the baseline changes a little. Repeated cycles of analyte binding followed by regeneration of the surface will provide insight into the overall performance of the surface. Ideally the surface performance should be evaluated for the same number of cycles that will be used during the SPR experiment.

The surface must be monitored for signs of accumulation and also degradation of the immobilized ligand (*Figure 4*). This can be accomplished by monitoring both the baseline at the beginning of each injection cycle and binding signal (slope or bound response) of a quality control sample. Appropriately defined acceptance criteria for system suitability such as baseline drift and quality control performance help to monitor the integrity of the immobilized ligand on the surface.

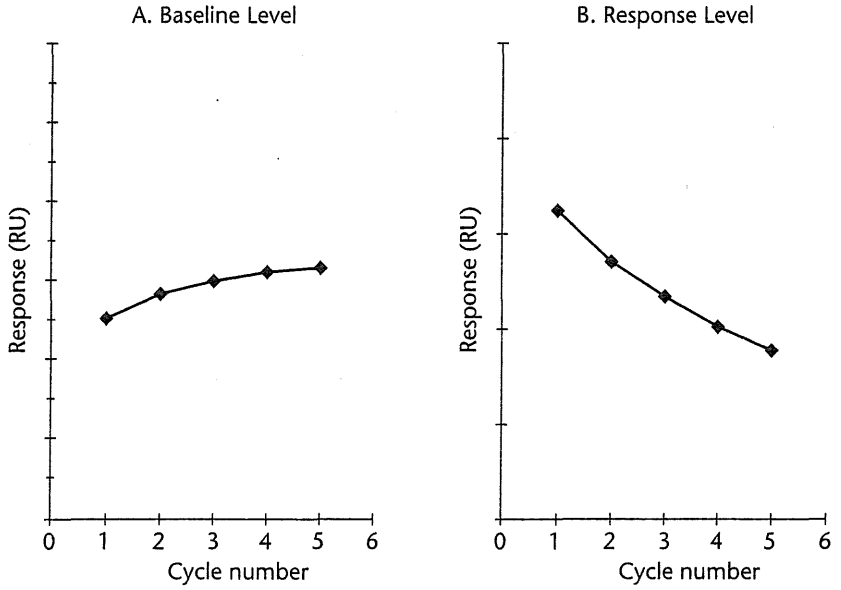

Figure 4. Evaluating surface performance (A) accumulation on surface and (B) degradation of immobilized ligand.

If the binding response is slowly decreasing, there are two possible explanations:

1. If the baseline of the raw data sensorgram remains constant but the binding response still decreases, the regeneration conditions cause an irreversible change to the biomolecule that decreases the binding capacity of the surface, which in turn decreases the amount of analyte that can be bound on the surface. Analysts can decrease the strength of the regeneration solution slightly or can change to another regeneration solution of equal strength within the same class.

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2. If the baseline increases, the accumulation of analyte causes a binding-capacity decrease on the surface, which in turn decreases the amount of analyte that can be bound to the surface. Analysts can increase the strength of the regeneration solution slightly or can change to a regeneration solution of equal strength within the same class. There may be a difference between regeneration solutions in their ability to solubilize the analyte.

Once a suitable regeneration solution has been determined, it should be tested in a series of analyte binding and regeneration cycles. Because the binding activity of the surface typically decreases with time and/or use, analysts must empirically determine the binding response threshold and consequent number of cycles for surface use. Examples for determining binding threshold and number of cycles are presented below (see Applications 1–3).

In some cases the baseline will drop or rise and/or the binding capacity will decrease somewhat in the first few injections before it stabilizes. This is caused by either the dissociation of electrostatically bound biomolecules from the surface (depending on surface characteristics) or to binding to a high-affinity non-regenerable fraction of the surface. For this reason, each newly immobilized surface should be conditioned with repeated analyte binding and regeneration cycles before collection of quantitative data. Alternative immobilization methods, such as a different chemistry or indirect capture, should be evaluated when the immobilized biomolecule is difficult to regenerate.

Data Analysis and Interpretation

Analysis and interpretation of the data are specific to the experimental objective. Several data analysis programs exist to aid in the calculation of kinetics and affinity constants from SPR data. The validity and quality of the results are linked directly to experimental design. The fitting process is purely mathematical, without regard to the biological significance of the values obtained.

Data Analysis Algorithm: Global analysis seeks a single set of kinetic rate constants for all of the analyte concentrations used in the experiment. Using a data-fitting algorithm such as Marquardt-Levenberg the data analysis software begins an iterative process starting with an initial approximation to find the best set of parameters that produces agreement between the experimental data (sensorgram) and the calculated fit to the data. The iterative process continues until the difference between the experimental and calculated (theoretical) curves is minimized as measured by the sum of the squared residuals.

Preparing the Data for Analysis: Before conducting kinetic analysis, analysts should inspect the experimental data visually for anomalies or artifacts such as baseline disturbances or out-of-range data (often due to air bubbles) lasting for a predefined time period (e.g., 4–8 s). Outliers should be removed from the data set according to pre-established criteria. Nonessential data, such as capture or regeneration injections, should be removed from the sensorgram, and the data at each analyte concentration should be adjusted using the double-referencing procedure described below. Before analysis the raw data should be processed in the following manner:

- Align the injection start to zero seconds for all concentrations and buffer injections for both the reference and active surfaces.
- Align the baseline to zero response for all sensorgrams.
- Subtract the reference surface sensorgram from the active surface sensorgram in order to create a corrected data set.
- Subtract the corrected buffer sensorgram from the sensorgrams at different concentrations in order to create a double-referenced data set.

The double-referencing procedure removes systematic errors (e.g., instrument noise) and low levels (less than 5% of total binding response) of nonspecific binding. It should not be used to correct for significant nonspecific binding events because this can lead to erroneous measurements.

When analyzing the data for kinetic information, analysts use the association (injection) and dissociation (buffer flow) phases for all of the concentrations in the series. For steady-state affinity analysis, the response at equilibrium R_{eq} (data plateau or no change in response vs. time) is measured for each sensorgram to create a binding isotherm with R_{eq} vs concentration. This isotherm is analyzed using the equations described below.

Kinetics and Steady-state Affinity Models: The Langmuir kinetic model assumes a 1:1 interaction between the binding partners so that

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

The association and dissociation rate constants are defined below:

$$\frac{d[AB]}{dt} = k_a \times [A] \times [B]$$

$$-\frac{d[AB]}{dt} = k_d \times [AB]$$

Combining these two equations and defining $[B_{free}] = [B_{tot} - AB]$, the net rate expression is

$$d[AB]/dt = k_a \times [A_{free}]\,[B_{tot} - AB] - k_d \times [AB]$$

which can be translated into terms from the SPR experiment as follows:

$$dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$$

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where R is the binding response at any point along the sensorgram and C is the known analyte concentration. Using global analysis as described above, k_a, k_d, and R_{max} are calculated from the experimental data using the rate equations shown below:

Association: $dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$

Dissociation: $dR/dt = -k_d \times R$

Because the concentration of analyte is zero during dissociation, the rate equation for dissociation depends only on the response, R, and the dissociation rate constant, k_d.

Application of the equilibrium condition where the complex formation (association) equals complex decay (dissociation)

$$k_a \times [A] \times [B] = k_d \times [AB]$$

yields the following equation for the equilibrium dissociation constant

$$K_D = \frac{[A]\times[B]}{[AB]} = \frac{C(R_{max} - R_{eq})}{R_{eq}}$$

where R_{eq} is the binding response at equilibrium that is measured in the experiment for a given analyte concentration and C, K_D, and R_{max} are calculated using global analysis.

Kinetics binding models can be used to describe non-1:1 interactions, e.g., bivalent interactions that occur if an antibody is used as the analyte, heterogeneity in binding partners, conformational change, or more complex interactions such as cooperative binding. SPR analysts are cautioned against using more complex models to assess data unless experimental design has been confirmed.

Assessing the Fit: The quality and validity of the fit to the kinetic data can be assessed by (1) visual inspection of the agreement between the experimental and calculated curves, (2) the size and the shape of residual plots, (3) the biological relevance of the results, and (4) statistical parameters such as χ^2 (average of squared residuals), and standard error (SE), T-value or U (uniqueness) factor. The best parameter fit to the experimental data should be superimposed on the curve for each concentration in the experiment. The residual plot visualizes the difference between the calculated and experimental data. The shape of the residuals should be random without trending (waviness or curving up or down). The height of the residual plot should reflect the instrument noise. Further, χ^2 should be minimized for a good fit with values that depend on the instrument noise, number of data points, and overall binding response. The parameter values should be considered for biological and experimental relevance. For example, is the calculated k_a value slow when the interaction is known to be fast, or is the calculated R_{max} value higher than the value that was calculated using Equations 1 and 2. Parameter significance is evaluated based on standard error, T-values, and U factor. Parameters that are significant cannot be changed without affecting the quality of the fit. All of the criteria should be within acceptable limits.

A similar set of criteria can be used for assessing the fit to steady-state affinity data, but because there are fewer data points (6–12 total, depending on the number of concentrations used), the statistical parameters and residual plots are less predictive of fit quality. Visual inspection of the agreement between the experimental and calculated binding isotherms and the parameter relevance are good tools to use for assessing the fit. Additionally, according to the relationship between concentration, K_D, and R_{max}, when concentration equals the K_D for the interaction R equals 50% of R_{max}. Confirming that the analyzed data follow this relationship provides another way to check the validity of the calculated result. When it is practical to calculate the K_D using both kinetic and steady-state analysis approaches, the K_D values should agree within experimental error.

Addressing a Lack of Fit: When the data do not fit or the parameter values do not make sense, often the problem can be resolved by a systematic approach that considers potential sources for deviations and tests each hypothesis. Items to consider include reagent purity, immobilization chemistry or surface density, analyte concentration errors, nonspecific binding, loss of ligand activity, or mass-transport–limited binding. Reviewing the raw (uncorrected) data helps determine the source of nonspecific binding or concentration errors. *Table 4* lists potential sources for deviations from 1:1 binding and recommended actions.

Table 4. Common Sources for Deviation from 1:1 Binding Kinetics

Source of Deviation	Recommended Action
Nonzero baseline before injection	• Normalize response to zero and reanalyze
Incorrect injection start and stop times or poorly defined injection start/stop	• Adjust injection start/stop • Remove sensorgram artifacts (e.g., injection or air bubble spikes)
Concentration input errors	• Verify concentration values and reanalyze
Bulk refractive index contribution too high	• Use double-referencing approach before analysis • Set RI = 0
Mass-transport–limited binding	• Vary flow rate (slow to fast) for a single concentration and overlay sensorgrams (should be identical for same association and dissociation time) • Include mass transport term, k_m, in fitting model • Reduce surface density

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Table 4. Common Sources for Deviation from 1:1 Binding Kinetics *(continued)*

Source of Deviation	Recommended Action
Nonspecific binding	• Change immobilization chemistry • Change sensor surface properties • Buffer additives—add or minimize • Reagent purity—repurify samples
Loss in binding partner activity	• Change immobilization chemistry • Change regeneration solution • Re-analyze data using local instead of global parameter fitting for R_{max}
Multi-valent binding interaction	• Immobilize multivalent binding partner

Recommendations for data analysis will be introduced in the subsequent sections of this chapter.

Application 1—Immunogenicity Assessment: SPR has emerged as a powerful technique for assessing immunogenicity of protein therapeutics. An advantage of this platform for detecting antibodies in serum (or plasma) samples is that it allows label-free detection based on mass accumulation in real time, which potentially allows detection of low-affinity antibodies of all classes and subclasses. This technology is useful both for screening assays (first-tier immunoassays that are used to detect the presence of antibodies capable of binding to a protein therapeutic) and also characterization assays. Characterization assays are useful for defining generated antibodies that bind to the protein and can include analysis of antibody concentration, isotype(s) represented, relative binding affinity, and binding specificity. A limitation is that SPR it is not appropriate for determination of the neutralizing capability of antibodies, which is best determined using cell-based biological assays. When designing and validating SPR assays for immunogenicity assessment, analysts should consider critical parameters including protein immobilization to ensure immunological reactivity, immobilized protein stability, and surface regeneration conditions.

Protein Immobilization: The first step in the development of immunogenicity assessment assays is to identify the optimum mechanism for immobilization of the target protein. When considering the target density for immobilization, analysts often recommend that a high-density surface be used. The advantage of a high-density surface is that it maximizes the opportunity that anti-therapeutic antibodies will come in contact with an immobilized ligand. A high-density surface also provides excellent assay sensitivity. An important aspect of these assays is that the chemistry chosen for immobilization should provide random orientation rather than a site-directed orientation so that all potential epitopes on the therapeutic protein are available for binding by the anti-therapeutic antibodies. The effectiveness of immobilization is determined by evaluating the ability of positive control antibodies to bind to the immobilized protein. When evaluating the effectiveness of immobilization, analysts should test multiple antibodies with different epitope specificities. When panels of antibodies that cover a range of affinities and bind to different epitopes on the target protein are all capable of binding, this provides confidence that antibodies contained in clinical specimens also will be detected. If any of the positive control antibodies do not demonstrate binding, this suggests that the immobilization is not optimal and should be modified. Although SPR is demonstrably efficient at detecting low-affinity antibodies, analysts should confirm that the immobilization protocol chosen is effective for detection of low- and high-affinity antibodies.

Protein Stability Upon Immobilization: The positive control antibody must be able to bind to the immobilized protein in order for an assay result to be acceptable. This confirmation of binding provides confidence that if antibodies against a protein are present in a sample, they will bind to the immobilized protein on the surface of the sensor. Because SPR relies on re-using the immobilized protein surface for multiple analyses, a regeneration protocol is required to effectively remove any bound material from the immobilized protein. This regeneration procedure is based on the ability to remove bound material without damaging or removing the immobilized protein. Several regeneration protocols can be used, and most often the regeneration solution is an acidic solution, commonly dilute HCl. The immobilized protein must remain intact and functional after repeated regeneration steps.

Because the immobilized protein will be used routinely for multiple analyses involving repeated cycles of serum samples, analysts should verify the stability of the immobilized protein after regeneration cycles. The stability of the immobilized protein can be monitored effectively by tracking the response units after regeneration and also after addition of positive control antibody. If there is a change in baseline or a decrease in the magnitude of binding by the positive control antibody, then the immobilized protein likely is no longer suitable for further analyses. The stability following regeneration should be established during assay development and should be confirmed during assay validation. In order to monitor the performance of the sensor during an assay, analysts should periodically test a positive control sample during an assay run. If the performance of the positive control samples indicates the immobilized protein has been compromised, analysts should re-analyze test samples obtained after the performance of the assay dropped below acceptable limits. Acceptance parameters for immobilization may vary by compound and should be established for each assay.

Availability of Epitopes After Immobilization: Once the protein is immobilized, the availability of multiple epitopes should be confirmed. Ideally this is done by testing for binding of positive control antibodies with different epitope specificity. One method for testing epitope availability is to use a panel of monoclonal antibodies that are known to recognize different regions of the protein. If the protein has been randomly immobilized, all the different positive control antibodies should be able to bind. The reason for evaluating epitope availability is to prevent false-negative results when serum samples are evaluated. If the immobilization is not random, it would be possible to consistently immobilize the protein via a specific epitope, thus making that epitope unavailable for binding by an antibody. Another possibility is that chemical modification of the protein to facilitate immobilization altered the protein's conformation.

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Surface Regeneration and Subsequent Protein Stability: Using the previous guidelines, analysts should monitor the surface for signs of accumulation and degradation of the immobilized ligand and discontinue use when necessary. For example, when the binding capacity of a positive control antibody (diluted in test serum) drops below 80% of initial capacity the surface should not be used.

Assay Cut-point Determination: When performing assays to determine if a serum sample contains antibodies against a protein, analysts sometimes observe a background level of binding. That background binding can vary depending on the nature of the immobilized protein and also the patient population being tested. In order to determine if a test sample contains antibodies, analysts compare binding to control samples that do not contain antibodies against the protein. A cut-point is established, and when a sample contains antibodies the binding is greater than that cut-point. Analysts determine the assay cut-point by analyzing a series of serum samples that do not contain antibodies against the immobilized protein and then performing statistical analysis to determine the level of binding consistent with a sample that does not contain antibodies. The cut-point should be established using the same conditions that will be used for sample analysis. Although different approaches are used for determining a cut-point, a common approach is to establish the mean from the binding of 50–100 serum samples from healthy volunteers and set the cut-point at 95% (equivalent to the mean plus 1.645 times the standard deviation for a normal distribution). Analysts should remove statistical outliers from the calculations because their inclusion can cause a high bias and raise the cut-point. This higher cut-point will result in identification of fewer samples with antibodies against the immobilized protein. The statistically evaluated cut-point is the response unit value that serum samples must exceed to be considered positive for the presence of antibodies against the therapeutic protein. An important feature of cut-point determination is that it may be different in different patient populations. For example, patients with inflammatory disease, may show a higher level of nonspecific reactivity compared to a normal population. This higher level of nonspecific binding would result in samples being identified as positive when they did not contain any antibodies specific for the protein. When this situation arises, predose serum samples can be used to establish a new patient population–specific mean and assay cut-point.

Analytical Procedure Development and Validation: Once the stability of the immobilized protein is confirmed, a regeneration procedure has been defined, and the cut-point established, the antibody testing method can be developed and validated. The conditions used for analyzing samples should be identical to those used to establish the assay cut-point. An important parameter to consider is the optimal dilution of the serum sample. Increasing the dilution factor reduces nonspecific binding by serum proteins but also reduces overall sensitivity. Most antibody assessment procedures use between 5% and 50% serum. As the percentage of serum that is tested decreases, the percentage of the binding signal that is due to nonspecific interaction also decreases, and subsequently the percentage of the signal mediated by antibodies binding to the immobilized protein increases. Besides dilution, other means to reduce nonspecific interaction include adding surfactants, increasing salt concentration, adding BSA or HSA, or adding soluble sensor surface support material such as carboxymethyl dextran or alginate to the dilution and running buffer.

Other important variables to optimize include flow rate and sample volume. The combination of flow rate and sample volume defines the contact time, the length of time during which a given sample is in contact with the immobilized protein. The longer a sample is in contact with the immobilized protein, the greater the chances for antibody binding. The next important aspect to consider is verification that initial binding is a result of an antibody and not some other serum component. This can be accomplished by adding an anti-human immunoglobulin reagent and monitoring subsequent binding. If the initial binding observed was due to an anti-protein antibody, this reagent will bind to that antibody (the anti-protein antibody remains bound to the immobilized protein). When a therapeutic monoclonal antibody is the immobilized protein, the confirmatory reagent must be screened and verified not to bind directly to the immobilized therapeutic monoclonal antibody. One option here is the immobilization of the Fab′ fragment rather than the intact therapeutic monoclonal antibody. The confirmatory reagent must be verified for specificity. Once all of the parameters are optimized, the assay can be validated. Validation parameters include those typically associated with immunogenicity assays (precision, specificity, sensitivity, and robustness) as well as parameters specific to SPR assays (protein immobilization, stability of immobilized surface, and number of regeneration cycles).

Interference by Serum Components: Depending on the immobilized protein, serum components other than antibodies specifically directed against the immobilized protein possibly could bind to the immobilized surface. It is also possible that serum components that block the ability of antibodies to bind to the immobilized protein could be present. Both of these can be evaluated by testing the binding of serum samples from the target subject population that are known not to contain antibodies against the immobilized protein and then monitoring to determine if any binding does occur. If nonspecific binding is identified, steps can be taken to reduce or eliminate it. These steps can include pretreatment of samples to remove the nonspecific reactant, addition of surfactant, or alteration of salt concentration in sample buffers to reduce nonspecific binding.

Analysts should verify that serum samples do not contain agents that are capable of inhibiting antibody binding to the immobilized protein (these could include soluble forms of the immobilized protein or soluble receptors that could bind to the immobilized protein and block binding of the antibodies to the immobilized protein). Analysts can add the positive control antibody to target serum samples and can evaluate binding. If binding is inhibited by the target serum samples compared with binding to normal human serum samples, steps can be taken to remove the inhibiting agent. Failure to identify target serum interference can result in either false-positive or false-negative results.

Implementation of Multiplex Assays: When a therapeutic protein is a second-generation product that has been modified from an original therapeutic protein (e.g., via pegylation or increased glycosylation), the presence of antibodies against both the original and the second-generation product should be evaluated simultaneously. This can be accomplished by immobilizing each protein on separate channels in the microfluidic device and allowing serum samples to bind in series or in parallel to both immobilized proteins. The rationale for testing for binding

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to both the original and the modified therapeutic protein is that antibodies generated against the modified protein could have binding specificity to the original protein as well. As part of the characterization of the immune response, analysts must understand the specificity of antibodies for both first- and second-generation products. When possible, binding to an endogenous counterpart might also be tested by immobilizing the endogenous protein on a separate flow cell or channel.

Characterization of Anti-Therapeutic Protein Antibodies: Once antibodies against a therapeutic protein have been captured by binding to the immobilized therapeutic protein, those antibodies can be characterized. The important features of anti-therapeutic antibodies that can be studied include the relative binding affinity, the amount of antibodies present in the serum sample, the isotype(s) of antibodies present in the sample, and binding specificity.

By monitoring the rate at which the response units decrease after the conclusion of sample addition to the sensor, analysts can determine the relative affinity of the antibodies. A high rate of dissociation is characteristic of a low-affinity antibody, and a slow rate of dissociation suggests the presence of high-affinity antibodies. It is useful to compare the dissociation rates with both the positive control antibody (typically a high-affinity antibody preparation) as well as a panel of monoclonal antibodies of known binding affinities.

The relative active concentration of antibodies present in a sample can be estimated by comparing the binding signal with the signal produced from a dilution series of the positive control. Analysts can generate a standard curve from the standard and can compare the active concentration of antibodies in the sample with that standard curve. Because the positive control does not exactly mimic the mixture of antibodies contained in the sample—in fact, the positive control is often obtained from hyperimmunized animals such as rabbits—the concentration value obtained is relative to the standard. This value only approximates the actual concentration of human antibodies. Because the same positive control can be used throughout clinical development, analysts can compare the amount of antibodies obtained from different subjects using this strategy. Because the instrument's signal is proportional to the mass that is binding to the sensor, this type of analysis provides value. Analysts should consider that IgM antibodies have five times the mass of IgG antibodies. Another approach for determining the concentration of antibodies is described in the concentration analysis section of this chapter (see Application 2 below).

The isotype of captured antibodies can be readily determined by monitoring binding associated with sequential addition of isotyping reagents. For example, if IgM antibodies are present and have bound to the immobilized protein, the addition of an anti-human IgM reagent will produce an additional signal. Isotyping reagents can be found with specificity towards IgM, IgG, IgE, IgA, IgG1, IgG2, IgG3, and IgG4. Because of steric hindrance, analysts may be required to repeat isotyping analyses in different sequences to be certain the presence of previously bound isotyping reagents has not hindered subsequent analyses. For example, assume a sample contains both IgG1 and IgG4 antibodies against a protein and both species have bound to the immobilized protein. Because the anti-IgG1 isotyping reagent has bound to the IgG1 antibodies, the isotyping reagent bound to them may prevent subsequent additions of an anti IgG4 reagent from binding to the IgG4, and the presence of the IgG4 would be undiscovered. Analysts will conclude that only IgG1 antibodies are present, but if the order of isotyping reagent addition were reversed the IgG4 antibodies would be discovered. This example underscores the importance of careful interpretation of isotyping results. This is a problem for subsequent analysis only if there is observed binding by a previous cycle of isotype reagent addition. The specificity of isotyping reagents should be confirmed before use. Analysts should, for example, verify that an anti-human IgG reagent binds only to human IgG and does not cross-react with human IgM.

The region of the therapeutic protein recognized by the antibodies can sometimes be determined by immobilizing versions of the protein that have been truncated, have point mutations, or contain only a fragment of the protein. If the antibodies fail to bind to the changed version of the protein, it suggests that the epitope toward which the antibody is directed was influenced by the change. It should be kept in mind that point mutations and truncations not only influence the primary sequence of a protein, but can also influence the tertiary structure (i.e. folding, conformation) of a protein. Also, a subject is likely to generate a population of antibodies with different specificities for a variety of epitopes. Despite this concern, the strategy just described can prove useful for identifying the region on the protein where the antibodies are binding.

Application 2—Concentration Analysis: SPR can be used to determine the concentration of biologics in defined buffer systems, e.g., eluates from purification columns, formulation buffers, and complex mixtures such as serum, fermentation broths, crude cell extracts, and cell suspensions. The concentration of an analyte is measured by its binding to the specific ligand or other molecules that can interact with any portion of the analyte. The analyte concentration is determined on a surface where the analyte-specific ligand or an analyte-specific capture reagent is immobilized. The binding rate or the mass of analyte bound is determined, and the analyte concentration is calculated using either a standard curve obtained from a concentration series of a purified and well characterized reference material or by a calibration-free analysis that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration.

Immobilization of Ligand: To determine concentration the ligand is immobilized covalently or non-covalently on the surface. Analysts select an appropriate coupling mechanism and chemistry to ensure the ligand's functional integrity. In order to provide conditions that favor partial or full independence of kinetic parameters, a high surface density of the ligand is desired.

A high-density surface allows the analyte to bind to the ligand under conditions that limit mass transport. The interaction between the analyte and the ligand can be described by the following two-step process:

$$A \xrightarrow{k_m} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$

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where A is the concentration of analyte in the sample, $A_{surface}$ is the concentration of analyte at the sensor surface, k_m is the mass transport coefficient, B is the immobilized ligand, AB is the analyte–ligand complex, and k_a and k_d are the rate constants for the reaction between A and B. The mass transport coefficient k_m depends on the flow rate, the dimensions of the flow cell, and the diffusion coefficient of the analyte. The analyte first must be transported from the bulk to the sensor surface to react with the immobilized ligand molecules. If this mass transport of the analyte is much faster than the association step between ligand and analyte, the overall observed binding will be driven by the kinetic rate constants of A and B, a prerequisite for accurately determining the kinetic parameters. If the mass transport of analyte is much slower than the association step, the binding will be limited by the mass transport process, and kinetic parameters for the specific interaction between A and B will be difficult to obtain. However, these conditions are desired for determining the active concentration of an analyte. A high density of ligand on the sensor surface and slower flow rates favor limited mass transport. Between these extremes, the overall binding is determined by contributions from both mass transport and interaction kinetics. It may not be possible to achieve limited conditions of mass transport for concentration analyses of interactions with relatively slow association rate constants (e.g., $k_a = 10^4\ M^{-1}s^{-1}$).

The ligand and the analyte reference material should be of sufficient purity with special attention to the presence of aggregated material. Aggregates of the analyte can interfere with the regeneration of the ligand surface because they can bind with multiple binding sites.

The reference material must be comparable (e.g., molecular weight and kinetic parameters) to the test samples. Under certain conditions the active concentration in unknown samples can be determined using a calibration-free procedure that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. These two methodologies are described separately below.

Concentration Determination with a Reference Standard Curve: In typical concentration-determination assays the analyte concentration is calculated from a standard curve that is obtained with a reference material injected at select concentrations. Three different approaches can be used to measure concentration with a reference standard calibration curve:

Direct Binding Assay: Determine the quantity of analyte bound after an arbitrarily fixed sample injection time. A sandwich method can be performed as an extension of the single-step direct binding approach in order to increase assay sensitivity.

Binding Rate Determination: Determine the initial binding rate for a sample rather than the amount bound. Under conditions of mass-transport limited binding, the binding rate is directly proportional to analyte concentration, and is independent of binding kinetics. This allows one to measure the concentration of related molecules that might have different binding characteristics.

Inhibition or Competition Assays: When the mechanism of action for an analyte is binding to a soluble ligand and thereby disrupting a ligand–receptor interaction, an inhibition assay can be used. In an inhibition assay, a receptor is attached to the sensor surface by a covalent linkage. The interaction between the analyte and the soluble ligand is indirectly measured by mixing a fixed concentration of ligand with varying concentrations of the analyte and injecting the ligand–analyte mixture across the immobilized receptor surface. Competitive methods in solution can also be used for large molecules and particles such as viruses, as well as for small analytes that give low direct responses. In parallel systems the assay can be designed so that the standard samples and the unknown sample are injected in parallel. This method can be useful for ligands that are difficult to regenerate.

Analysts can plot the signal (amount bound or rate of binding) of the reference material standards against concentrations and then can generate a standard curve using an appropriate mathematical model such as a linear or a logistic four-parameter curve fit. Samples can be injected at one or more dilutions. Fewer dilutions can be employed if a linear relationship between sample and reference standard has been demonstrated. Concentrations of unknown samples are either obtained by back-calculation from the standard curve or, if they are analyzed at the same target concentrations as those of the reference standard curve, by comparison of curve-fit parameters.

Parameters that can influence assay performance and results include but are not limited to flow rate, ligand density on the surface, sample purity, sample matrix, and reproducibility of surface regeneration. These parameters must be evaluated during assay qualification or validation. Interference with binding of analyte to the immobilized ligand can be minimized by salts, detergents, or sensor-surface support material. A commonly used sensor surface consists of carboxymethylated dextran, so the addition of dextran to the sample dilution buffer can minimize nonspecific interactions. Injections over a negative control surface can also be used to mathematically subtract the nonspecific binding data from the data obtained on the positive surface. A qualified or validated concentration determination SPR assay should include QC samples that can serve as measure to determine the accuracy of the standard curve that has been prepared to analyze samples with unknown analyte concentration. They can be conveniently prepared in larger batches, qualified for use with a Certificate of Analysis for the target concentration, and stored in small aliquots under appropriate storage conditions.

After each analyte injection the ligand surface is regenerated and all bound analyte is removed. This regeneration must be strong enough to remove all bound analyte, but the conditions also must leave the immobilized ligand intact so that injections can be compared to each other.

Concentration Determination Without Calibration: Calibration-free concentration assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. By measuring the initial binding rate analysts can derive the analyte concentration if specific properties of analyte and the analytical environment are known. This approach can be useful when no satisfactory reference standard is available.

To determine the analyte concentration in a sample, analysts use the relationship between initial binding rate and analyte concentration. On a sensor surface with a high immobilization level, the initial binding rate (slope) can

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be described as a function of the molecular weight, the mass transport coefficient k_m, and the concentration of the analyte. Before a sample is analyzed analysts must determine the mass transport coefficient. It depends on the diffusion coefficient (D), flow rate, and flow cell dimensions and is described by the following formula:

$$k_m = 0.98 \times \sqrt[3]{\frac{D^2 \times f}{0.3 \times h^2 \times w \times l}}$$

where D is the diffusion coefficient, f is the flow rate, and h, w, and l are the flow cell height, width, and length, respectively. Flow rate and flow cell dimensions typically are known for a given instrument, and the diffusion coefficient is determined by the size and shape of the molecule by the use of instrument-specific tools, literature references, or experiments, e.g., by analytical ultracentrifugation or light scattering.

In a typical experimental setup the evaluation requires two flow rates. By using measurements at two widely separated flow rates, analysts can assess the influence of flow rate on binding rate. The robustness of the assay is also improved by fitting the data obtained at two different flow rates, which give correspondingly two different values for k_m (because k_m depends on the flow rate), to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

Calibration-free concentration analysis is suitable only for proteins with MW ≥ 5000 Da. It requires fast analyte–ligand association ($k_a > 5 \times 10^4$ $M^{-1}s^{-1}$) and it cannot handle mixtures of analytes with different diffusion properties. The dynamic range of the method is approximately 0.05–5 µg/mL.

Application 3—Kinetic and Affinity Analysis: Because of its ability to detect binding interactions in real time, SPR provides valuable information about the kinetics of complex formation and dissociation. SPR instruments can be used to determine the association rate constant and dissociation rate constant for a particular binding interaction, and these values can be used subsequently to calculate the dissociation equilibrium constant ($K_D = k_d/k_a$). Obtaining K_D from a ratio of k_a and k_d is useful when the binding interaction does not reach equilibrium in a timeframe that is suitable for an SPR binding experiment. For binding interactions that reach equilibrium (rate of complex formation equals the rate of complex decay) in min (vs. h), K_D can be determined directly from a steady-state binding response. The length of time required to reach equilibrium is influenced by the dissociation rate, so quickly dissociating complexes (e.g. $k_d = 10^{-2}$ s^{-1}) will reach equilibrium faster than those that dissociate slowly (e.g., $k_d = 10^{-5}$ s^{-1}). Software programs capable of simulating 1:1 binding kinetics are useful for predicting the length of time required to reach equilibrium. The typical working range for affinity measurements with commercially available SPR instruments is from 10^{-12} M (pM) to 10^{-4} M (µM).

Proper experimental design is required to accurately measure k_a, k_d, and K_D. Several questions must be considered when designing kinetic analysis or steady-state affinity experiments, including:

- Which binding partner should be immobilized?
- How will the analyst immobilize one of the binding partners?
- What type of reference surface should be used?
- How much binding partner should be immobilized?
- Does the binding partner maintain activity after immobilization?
- Is binding to the immobilized binding partner specific?
- What regeneration conditions, if necessary, should be used?

When selecting which binding partner to immobilize for most protein–protein interactions, analysts must consider several factors: (1) the purity and availability of the proteins, (2) the presence of a tag or functional group to aid in immobilization, (3) maintaining biological activity, and (4) the binding valency (e.g., monovalent vs. multivalent binding).

A reference surface is required for all detailed kinetic and affinity analysis experiments. If direct immobilization is used, then a reference surface is created using the same immobilization protocol, omitting the protein during the coupling step. Alternatively, a mutant form of the protein with a modified binding site can be used. The reference surface for high-affinity capture typically consists of either the capture molecule and no binding partner, or uses an unrelated molecule for a mock capture surface. For the specific case of antibody–antigen interactions, an unrelated monoclonal antibody often serves as the capture reagent on the reference surface.

After deciding on the immobilization approach, analysts must decide how much binding partner to immobilize. For kinetic analysis, the primary consideration is to minimize the surface density to avoid mass-transport–limited binding of the analyte molecule to the immobilized binding partner. Analysts also must consider the immobilization level when conducting steady-state affinity analysis because high immobilization levels can cause steric hindrance or can induce secondary effects such as nonspecific binding or aggregation.

Before performing a kinetic experiment or steady-state affinity analysis, analysts must assess the activity of the surface by injecting the analyte molecule at a single concentration. The concentration should be high enough that the equilibrium binding response provides a close approximation of the experimental maximum response (R_{max}). This condition is typically met when the target molecule concentration is at least 10-fold higher than the K_D of the binding interaction. For a protein–protein interaction having a K_D value of 100 pM this means that the target molecule should be injected at a concentration of at least 1 nM. Using the R_{max} equation (*Equations 1* and *2*), analysts can calculate the theoretical R_{max} based on the amount of binding partner immobilized or captured. If the experimental R_{max} exceeds the theoretical R_{max}, then the analyte molecule is larger than expected or the analyte exists in a higher-order structure than expected (e.g., following aggregation or as a multimer). If the experimental

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R_{max} is significantly lower (<50%) than the theoretical R_{max}, this suggests that the immobilization procedure has compromised the binding site, and an alternative immobilization procedure should be investigated. An advantage of using high-affinity capture instead of direct coupling is that the surface activity typically remains close to 100%, provided that the specific activity of the immobilized binding partner is 100% before capture.

Injecting the target molecule across the reference surface also provides a quick assessment of the amount of nonspecific binding that exists on the sensor surface. Nonspecific binding that is electrostatic in nature can be eliminated or reduced by addition of salt (e.g., 0.5–1.0 M NaCl) to the sample diluent buffer and the running buffer or by using sensor surfaces with a low charge density. Nonspecific binding that arises from hydrophobic interactions can be minimized or eliminated by the addition of detergents such as 0.05% Polysorbate 20 or 10 mM CHAPS to the sample diluent buffer and the running buffer. Before using buffer additives, analysts should test whether the specific binding interaction or binding activity is affected. Nonspecific binding to a capture molecule can be resolved by switching to a different capture molecule. Reducing surface density also may eliminate nonspecific binding.

Many protein–protein interactions dissociate slowly ($k_d = 10^{-3}$ to 10^{-6} s^{-1}), with complex half-lives ($t_{1/2}$) of more than 2 h. A regeneration step is important for these types of binding interactions.

In some protein–protein interactions, surface regeneration may not be possible because of a high-affinity binding interaction between the molecules. If this situation occurs, analysts can consider a titration kinetic experiment or the use of instruments that are capable of performing parallel analyte injections. In a kinetic titration, increasing concentrations of target molecule are injected consecutively across the immobilized surface, and the resulting data are analyzed using a titration kinetics model. In parallel instruments the analyte concentration series is injected in one step, thus eliminating the need for regeneration.

Surface regeneration typically is not performed for steady-state affinity experiments because the binding interactions in this type of experiment have k_d values in the range of 10^{-2} to 0.5 s^{-1}, and therefore have $t_{1/2}$ values of <2 min. The bound analyte dissociates from the surface as buffer flows over the surface, and regeneration is not required.

Analysts should have an accurate analyte concentration for kinetic analysis because the calculation of k_a depends on the analyte concentration. Typically an absorbance reading at a wavelength of 280 nm (A_{280}) is used for this purpose, but analysts should remember that the A_{280} value reflects the total bulk protein in solution and does not reflect the actual concentration of protein that is capable of binding (i.e., the active concentration).

Sample diluent injections should be included in replicate for kinetics and steady-state affinity experiments so that nonspecific responses due to instrumentation or sample diluent can be removed during the data-evaluation process.

The recommended analyte concentration range is 10-fold below and above the K_D for the interaction. By keeping the surface density low (R_{max} = 5–50 RU) and extending the association time, analysts should be able to collect data with enough curvature to accurately define k_a and k_d values. When the affinity of the interaction is high (K_D = low nM to pM), higher analyte concentrations may be required to build a kinetic profile with sufficient curvature to complete the kinetic analysis. Increasing the analyte concentration should not be a substitute for changing other experimental design parameters (e.g., surface density and contact time). Although it is desirable to use analyte concentrations that approach R_{max}, unusually high concentrations may induce aggregation of the analyte in solution or nonspecific binding to the surface.

Within an analyte concentration series, replicate samples should be used to assess surface activity. Additionally, each concentration series should be tested 3–5 times using different surfaces in order to establish confidence intervals for the resulting kinetics and affinity constants.

The amount of data that is collected during the kinetic or steady-state affinity experiment affects the accuracy of the results. For kinetic experiments involving binding interactions with slow k_d values, analysts should collect enough dissociation data so that a measurable dissociation response (vs. instrument noise) is acquired. For example, a binding interaction between a therapeutic monoclonal antibody and its target molecule that has a k_d of 10^{-5} s^{-1} will require the collection of at least 4 h of dissociation data. Rather than collecting this much dissociation data for all analyte concentrations, analysts can use a long dissociation time for the highest concentration of analyte that will be injected, and they can use a short dissociation time (2–5 min) for all other analyte concentrations. With parallel-injection instruments one can collect the long dissociation data for all of the concentrations. For data evaluation analysts should collect both long and short dissociation time data for sample diluent injections. For steady-state affinity experiments, the analyte injection time should be long enough to allow for a steady-state binding response to occur at all analyte concentrations that are tested. Dissociation data do not have to be collected for a steady-state affinity experiment because dissociation data are not used in the evaluation of this type of experiment, but the complete dissociation of analyte is required before beginning the next injection.

Use of SPR in a Regulated Environment

When SPR assays are used for lot release and stability testing, the assay must exist within a controlled setting so that decisions can be made about the use of product within the clinic or marketplace. SPR instrumentation, including software, should be 21 CFR Part 11 compliant and should be amenable to validation. These requirements are important because they help ensure the integrity of both data acquisition and data evaluation.

Besides using SPR instrumentation that meets regulatory requirements, analysts should establish system suitability criteria for an SPR assay. Including these criteria in an SPR assay ensures that the results obtained for the test sample are generated by an assay that is performing within its operating parameters. A discussion of assessing system suitability parameters is not within the scope of this chapter, but the reader is referred to USP general chapters *Design and Analysis of Biological Assays* ⟨111⟩ and *Analysis of Biological Assays* ⟨1034⟩ for more detailed discussions. Some examples of system suitability parameters for an SPR assay can include:

- ligand immobilization density
- parameters from a curve fitting model (e.g. four parameter logistic curve fit)

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  - EC_{50} values for reference standard curve and positive QC curve
  - Effective asymptotes (response range) for reference standard curve and positive QC curve
  - R^2 values for reference standard curve and positive QC curve
  - parallelism between reference standard curve and positive QC curve
- relative bioactivity for positive QC (EC_{50} ratio of reference standard to positive control)
- calculated concentration for positive QC (single-point positive QC measurement)
- binding response for negative QC (nonspecific analyte or diluent)

Multiple lots of ligand, analyte, coupling reagents, and sensor surfaces should be used to establish assay system suitability criteria that reflect normal assay conditions. Assay results for reference standard and QC samples should be tracked over time. Regular trending analyses should be done on the data to show whether the SPR assay remains in control over its required lifecycle. If trending in the data is observed, remedies can be performed proactively, preventing assay failure.

For SPR assays that are used in lot-release testing, it is also important to establish sample acceptance criteria. These criteria are used to accept or reject sample data and can include:

- relative bioactivity for test sample
- coefficient of variation (CV) for sample replicates
- parallelism between reference standard curve and positive QC curve

Another point to consider for SPR assays in regulated environments is the identification of noncritical and critical reagents. Noncritical reagents typically include coupling buffer, regeneration buffer, and continuous flow (running) buffer. Critical reagents typically include the ligand and analyte for direct binding assays, and ligand, analyte, and competitor molecule in inhibition binding assays. Critical reagents should be qualified/requalified on a routine basis to ensure that they are suitable for use in the SPR assay. Best practices for the characterization of critical reagents are not within the scope of this chapter, so the reader is referred to current regulatory documents for such discussion. Analysts should use current regulatory documents such as ICH Guideline Q2(R1) Validation of Analytical Procedures and *USP–NF General Chapter Validation of Compendial Procedures* ⟨1225⟩ when they validate SPR assays in a regulated laboratory.

⟨1106⟩ IMMUNOGENICITY ASSAYS—DESIGN AND VALIDATION OF IMMUNOASSAYS TO DETECT ANTI-DRUG ANTIBODIES

INTRODUCTION AND SCOPE

Anti-drug antibodies (ADA) can be induced when animal or human immune systems recognize a protein drug product as foreign. The administration of biopharmaceuticals can elicit product-specific ADA, and various types of ADA responses can develop in either nonclinical or clinical studies. [NOTE—A list of regulatory documents, white papers, and other relevant references is contained in the *Appendix*.] Although the main focus of this chapter is ADA immunoassay design and validation, the chapter also includes discussion of an overall risk-based immunogenicity assay testing strategy that includes preclinical and clinical studies.

ADA assay results are directly influenced by assay design, assay reagents, how the assay is run, what samples are run in the assay (timing of sample collection, etc.), and how assay data are analyzed. In fact, it essentially is impossible to compare the results of studies that use different ADA assays. Guidance, such as this general information chapter, recommending best practices and considerations for ADA assay development helps ensure that the assays produce results that are meaningful for patient safety and product efficacy.

The primary concern with unintended or unwanted immunogenicity of biological products is whether antibodies produced by patients receiving the product lead to some form of clinical response (e.g., an effect on safety or efficacy). The utility and interpretation of preclinical toxicology studies also can be influenced by the presence of ADA.

The pharmacokinetics (PK) or the pharmacologic activity of the drug can be altered by ADA that either enhance or reduce the clearance of the drug, alter bioavailability, or inhibit or exacerbate the pharmacological action of the drug. If an endogenous counterpart of a drug exists, ADA that inhibit the activity of the product also can bind to and cross-react with an endogenous protein counterpart of the product, potentially leading to a deficiency syndrome. Under some circumstances, ADA can form immune complexes that can induce serum sickness-type clinical responses. Moreover, IgE isotype ADA responses can result in anaphylaxis.

Immunogenicity assessments are playing an increasing role in biopharmaceutical development as part of Product Quality Risk Assessments (PQRAs) and the assessment of the criticality of quality attributes (as described in ICH Q8 and Q9). They also play a role in the demonstration of process and product comparability after manufacturing process changes. Often the manufacturing process for a biological therapeutic will be refined during clinical development, and often changes occur after the sponsor obtains marketing authorization. Such changes, however minor, potentially could affect the bioactivity, efficacy, or safety of a biotherapeutic, and immunogenicity is a key consideration. Changes in the levels and types of degradation products (oxidized, deamidated, aggregates, or others), isoforms of the protein, and process-related impurities such as host cell protein and DNA could affect immunogenicity and warrant closer evaluation.

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FACTORS THAT AFFECT THE IMMUNOGENIC POTENTIAL OF A THERAPEUTIC PROTEIN

Many factors can influence whether administration of a biological product will induce an immune response in the recipient, including the structure of the protein product itself, product variants, product formulations, the immune status and genetic makeup of the patient, and the dosing route and regimen used in the clinic.

Protein Structure

The primary amino acid structure of the product and its variants can determine if there are immunogenic epitopes that the patient's immune system recognizes as foreign, leading to an immune response. Amino acid sequences that are not found in human proteins and thus could be recognized as foreign by the human immune system (e.g., those derived from a nonhuman cell line or created by protein engineering, e.g., fusion proteins) not surprisingly can induce an immune response in humans. In addition, chemical modification of amino acids (e.g., oxidation or deamidation) may result in a sequence that can stimulate an immune response, although few data to date have confirmed such occurrences following administration of therapeutic proteins. Truncation of the protein could expose amino acid motifs (neoepitopes) not normally exposed in the native protein, stimulating an immune response.

In general, glycosylation does not appear to play a major role in the induction of immune responses to biological products, although nonhuman (e.g., murine) or nonmammalian (e.g., products derived from plants) glycosylation can induce immune responses. An example is a human monoclonal antibody that contained a terminal galactose-α-1,3-galactose because of posttranslational modification by its murine production cell line. This antibody was antigenic and caused severe hypersensitivity reactions in presensitized individuals bearing cross-reactive IgE. There was no evidence that this glycan induced primary immune reactions in naïve (i.e., not presensitized) individuals. Such examples are rare. In fact, in many cases complex carbohydrates may prevent or reduce the antigenicity of immunogenic proteins by shielding epitopes from binding antibodies.

Aggregated protein has been shown to induce immunogenicity in animal models. Proposed mechanisms of action include either presentation of high molecular weight, repeating subunits (multimers) directly to B-cells thereby inducing a T-cell–independent response or by inducing a T-cell–dependent response via enhanced antigen presentation. Addition of polyethylene glycol (PEG) molecules (PEGylation) to recombinant therapeutics has been attempted in order to attenuate immunogenicity and antigenicity of recombinant proteins by limiting exposure of epitopes. Although no clear evidence establishes that the immunogenicity of proteins is diminished by PEGylation, some clinical data suggest that PEGylation can limit antibody binding (i.e., antigenicity) to the protein backbone. PEG itself can be immunogenic and anti-PEG antibodies should be monitored, especially in subjects with known PEG hypersensitivity. In fact, a background level of anti-PEG antibodies is present in the general population, leading to the requirement to develop an anti-PEG antibody assay as well as an ADA assay during the clinical development of PEGylated products.

Process-Related Impurities

Process-related impurities such as endotoxins, host cell DNA, or proteins can act as adjuvants and can provoke an immune response by evoking danger signals via activation of immune cellular receptors such as Toll-like receptors. Some postulate that leachables from primary packaging components also can act as immune stimulators or affect the higher-order structure of the protein product and induce an immunogenic response, although firm data are not yet available.

Immune Status of the Patient

The ability of the patient's immune system to recognize and respond to a protein product can dictate the level of immune response. Patients who are taking immunosuppressive drugs such as glucocorticoids, cyclosporine, or methotrexate may have a lower likelihood of immune response to a protein product despite its immunogenic potential. Conversely, autoimmune diseases and inflammation may involve the overactivation of an immune system so that a product's level of immunogenicity may be much greater than one would anticipate. The pharmacologic activity of the protein therapeutic itself should also be taken into account. Some protein therapeutics directed against B-cell antigens can deplete peripheral B-cells. Conversely, other protein therapeutics may have immune-modulatory activities, e.g., altering patterns of T-cell trafficking. These activities may affect an individual patient's or a patient population's ability to mount an immune response to a protein product.

A patient's immune status should also be considered when the patient exhibits specific pre-existing ADA, cross-reacting ADA (for example, in the case of murine- or plant-derived products), or antibodies against production cell line-related impurities that might induce a clinical response.

Genetic Background of the Patient

Molecules that recognize and present protein-derived peptides to the adaptive immune system—the human leukocyte antigen or major histocompatability antigen molecules—show considerable genetic diversity between individuals and between populations in different parts of the world. This diversity is one reason why different patients may have different immune responses to the same product. Consequently, if clinical studies include only a population of limited genetic diversity, then the immunogenicity profile of a protein therapeutic in that population may not reflect its immunogenicity profile in the larger, more diverse population that would be exposed to product after approval.

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Dose and Route of Administration

The way a therapeutic protein product is used can influence its potential for immunogenicity. Different routes of administration appear to have different effects on immunogenicity, and subcutaneous injection generally is perceived to be a more immunogenic route of exposure than is intravenous administration. The dosing regimen also can influence immunogenicity. A protein therapeutic that typically is administered one time as a single dose (e.g., a thrombolytic protein) is less likely to induce an immune response than is a protein therapeutic that is used in a multidose regimen because the immune system usually requires a prime followed by a boost to ensure a robust response. Patients may have pre-existing sensitization even without any known exposure to a therapeutic protein product, and they may exhibit adverse clinical responses on first exposure. However, products with long half-lives or those that are particularly immunogenic (e.g., those with multiple T-cell epitopes) have been shown to induce ADA after a single injection. A chronic dosing regimen has a greater chance of inducing an immune response because the immune system receives multiple exposures to the product and this can lead to a strong memory T-cell response.

Another regimen that has caused ADA with clinical sequelae is one whereby a product is given for a short period of time, stopped, and then introduced again only after a long lag period. This has the effect of priming the immune system, and the reintroduction can cause immune-related events such as allergic responses. The use of chronic dosing on a regular basis, although it repeatedly provides the drug product, appears to avoid such hyper-responsiveness by inducing some form of tolerance.

DETERMINATION OF PRECLINICAL AND CLINICAL IMMUNOGENICITY

Preclinical: Relevance and Scope of Preclinical Immunogenicity

The immunogenicity of many biotherapeutics is greater in preclinical studies and has low predictive value for humans because an immune response to human or humanized proteins tends to be greater in animals than in humans because of the perceived foreignness of the protein in animals and the absence of an endogenous biological counterpart. Even though detection of ADA in animals may not be clinically relevant, researchers must assess ADA for the interpretation of the required toxicity data necessary for regulatory submissions (see ICH S6R1 in the *Appendix*).

Generally ADA assessments, PK data, and pharmacodynamic (PD) data aid in the interpretation of the results and validity of animal toxicology studies. In some instances preclinical immunogenicity data also can be used to compare the relative immunogenicity of products before and after manufacturing changes, although only with the grossest of changes in relative immunogenicity does this appear to be meaningful. Preclinical studies, particularly in higher animal species, are typically not statistically powered to draw conclusions on relative immunogenicity but, when they can, it should be recognized that differences in MHC restriction and natural immune tolerance of animals versus humans may not permit translation of such information to effects in humans. Furthermore, it should also be acknowledged that strains of preclinical species are limited in genetic diversity compared to the human population.

ADA may alter exposure to an active drug by blocking the therapeutic agent from binding to the target or by accelerating the clearance of the therapeutic agent from circulation, resulting in reduced exposure. ADA may increase the half life of biologic drugs and with this the exposure if the drug–ADA complex is still bioactive. Typically, samples should be collected for possible ADA analysis from all preclinical safety studies in which animals are exposed to the drug for more than 7 days (see references in the *Appendix* for more information). Because the key consideration for including immunogenicity analysis in nonclinical studies is to demonstrate the exposure to the drug for the duration of the study, this also can be achieved by demonstrating drug-mediated modulation of a PD marker. In addition, an absence of an effect on the serum concentration or PK profile of the drug also can indirectly demonstrate the absence of detectable effects of an ADA on drug exposure. However, in some situations the development of an ADA may not significantly affect the clearance of the drug, but instead the ADA may affect the drug's binding to and activity at the target (e.g., anti-idiotype antibodies). Therefore, lack of an effect on a PD marker and/or on PK also should be considered to ensure activity is not affected by the ADA.

For most nonclinical studies, samples from various phases of the study should be collected, banked, and analyzed with regard to any observed pharmacological or toxicological changes. In fact, most nonclinical toxicology studies do not evaluate the kinetics of ADA development, and samples for the assessment of ADA usually are taken at baseline, end of treatment, and end of recovery periods.

Analyses of samples taken during the dosing phase also can be performed if unusual PK data or toxicological findings are observed at the end of the study. In this case it needs to be taken into consideration that analysis of samples taken during the dosing phase may be complicated by drug interference. Therefore, it is important to take samples at appropriate time-points (e.g., before the next dose) and to have suitable assays with high drug tolerance in place. In some instances when a soluble target may be present in the circulation, understanding of ADA level (titers or relative mass units) can facilitate the interpretation of toxicological findings.

A risk-based approach should be used to determine if further characterization (e.g., demonstrating neutralizing activity) is necessary for preclinical studies. Factors such as the presence of endogenous counterparts, utility of a PD marker, or the PK assay may affect this decision. If data from a neutralizing antibody assay (NAb) are deemed necessary, then this assay could be either a target-binding inhibition immunoassay or cell-based assay, irrespective of the risk level. Immunogenicity evaluation and study data interpretation typically require serial sampling and analysis of serum samples for PK, PD, and ADA. Such repeated and frequent sampling may not be feasible when researchers conduct studies using rodents, particularly mice. In such cases, the study can be designed to allow discrete analyses of toxicity, PK, PD, and ADA endpoints from similarly treated cohorts of mice with sample collection at similar study time points to allow inferential analysis of effects observed across treatment groups.

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Clinical: Relevance and Scope of Immunogenicity Assessments

In clinical studies, ADA detection and characterization is important to understand the safety and exposure and efficacy profile of the therapeutic. Typically, the ADA analysis strategy in clinical studies involves a screening assay for binding antibodies then a confirmatory assay, followed by further characterization in NAb. Immunogenicity data from clinical studies generally are analyzed in the context of their relevance to the PK and PD of the therapeutic and on adverse events. For replacement therapies (e.g., enzyme-replacement therapies, blood clotting factors, and erythropoietin), a comprehensive monitoring program should be designed based on ADA detection combined with multiple safety parameters to monitor the potential for serious adverse events.

Researchers should consider the kinetics of the appearance of ADA during clinical studies because this can affect not only ADA detection but also the potential clinical sequelae. Some products induce antibodies rapidly, but other biotherapeutics can take years before an immune response is detected or can be correlated to any clinical sequelae. Investigators also should consider whether an antibody response is transient or persistent. Therefore, understanding the kinetics of ADA appearance is important. This goal can be achieved by carefully selecting ADA sample collection times and taking care in developing the ADA assay for clinical studies. Samples should be collected during each phase of the study (pretreatment, during treatment, and during any washout phases). The sensitivity and drug tolerance of the ADA assay also must be appropriate for the intended use of the assay. For example, when products have long terminal half-lives (e.g., monoclonal antibodies) scientists should develop ADA assays that are capable of detecting ADA in the presence of product levels that are expected to be present in patient test samples. In addition, during the design of ADA sampling plans, researchers should consider the appropriateness of obtaining samples after a washout period.

The number of patients assessed for ADA in clinical trials and the duration and timing of ADA sample collection are critical factors to understand the incidence and clinical impact of ADA. Other factors may confound ADA analysis, including the nature of the therapeutic itself, the presence of pre-existing cross-reactive antibodies, rheumatoid factors, heterophilic antibodies, soluble targets, or ligands. Samples should be taken to assess the levels of these interfering factors in serum (and other PD markers as applicable) at the same time as ADA samples.

Besides determining the presence of binding antibodies, clinical immunogenicity assessments typically include further characterization of positive samples in titer assays as well as in NAb to determine the potential effect of ADA levels to neutralize the drug's effect or to mediate safety events. In addition, understanding the kinetics of ADA and NAb development by detection of ADA in sequential samples taken throughout the study phases and elucidation of the ADA immunoglobulin class(es) and subclass(es) may aid in better understanding of patient- and treatment-related factors and the mechanisms by which the therapeutic induces ADA development.

RISK-BASED APPROACH TO ASSESSING IMMUNOGENICITY AND ITS CONSEQUENCES

Assessing the Potential Risk of Product-Specific Immunogenicity

The concept of risk is defined in ICH Q9 as the combination of the probability of occurrence of *harm* and the *severity* of that harm. In relation to pharmaceuticals, the protection of the patient by managing the risk to safety should be considered of prime importance and thus risk assessments of product-induced immune responses should focus on the potential severity of clinical consequences from ADA responses rather than the probability of occurrence of ADA responses. A few patients with severe or life-threatening ADA-related side effects are of more concern than many ADA-positive patients who have no clinical consequences. Risk mitigation should also be factored into the overall risk-assessment process (e.g., elimination of clinical impact by co-medication).

Although ADA testing strategies can be based on immunogenicity risk assessment, this may not always be feasible during early drug development when reliable assays may not be available.

ADA-induced safety events can range from mild side effects to life-threatening conditions. The potential severity of the consequences of an ADA response should be considered as early as possible. *Table 1* summarizes some but not all of the risk factors that may influence the severity of clinical consequences from an ADA response.

Table 1. Factors That May Influence the Severity of ADA-Related Clinical Sequelae

	Lower Risk[1]	Medium Risk	Higher Risk
Presence of an identical endogenous counterpart (includes families of proteins that share domains)	No endogenous counterpart (e.g., Botulinum toxin)	Redundant counterpart (e.g., interferons or growth hormones)	Nonredundant counterpart [e.g., erythropoietin or megakaryocyte growth and development factor (MGDF)]
Presence of a structurally related endogenous molecule or domain	No structurally related endogenous molecule or domain	Medium structurally related endogenous molecule or domain exists	Highly structurally related endogenous molecule or domain exists
Patient's disease state	Life-threatening	Moderate-to-severe	Mild
Potential clinical consequences of immunogenicity	No clinically meaningful impact on safety or efficacy	Manageable impact to safety and/or efficacy	Extensive clinical influence on safety or loss (or dramatic increase) of efficacy

[1] Risk is a consequence of both severity and frequency.

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A number of factors may contribute to the incidence of an ADA response, and some but not all of these factors are shown in *Table 2*. During an immunogenicity risk assessment the factors shown in *Table 2* should be considered in conjunction with those in *Table 1*. The clinical consequences of immunogenicity are unpredictable, even with the risk assessments outlined above.

An immunogenicity risk assessment is of real value only when all the factors that influence the likelihood and severity of a potential immune response are carefully considered. Risk assessments should be done in a cross-functional manner, including input from clinicians, safety assessment, PK, bioanalytical scientists, as well as process scientists. Consultations with regulators and clinical safety monitoring boards also may be helpful and should be carried out iteratively during the product development process as clinical data are obtained.

ADA-mediated clinical sequelae and ADA incidence rate are separate entities, but the two factors are interrelated because the number of patients with ADA-mediated serious adverse events may rise with a higher ADA incidence rate.

Table 2. Factors That May Influence ADA Incidence

	Lower Incidence	Medium Incidence	Higher Incidence
Circulating level of endogenous counterpart	Abundant	Scarce	None
Patient's immune status	Suppressed	Normal	Activated
Exposure: dosing regimen or frequency	Single dose	Chronic (maintenance)	Episodic dosing
		Multiple dosing	
Route of administration	Intravenous or oral	Subcutaneous, intramuscular, mucosal (non-inhalational)	Intradermal or inhalational
Product Characteristics	Lowest levels (or absence) of product or process-related impurities (e.g., aggregates or denaturation, fragmentation)	Intermediate levels of product- or process-related impurities, and potential epitope content	Higher levels of product- or process-related impurities (e.g., aggregates; denaturation, fragmentation)
	Molecular integrity of active substance maintained		Higher level of antigenic epitopes (derived from murine cell lines, contains novel mutated sequences, etc.)
	No or low content of potential T-cell epitopes		Mechanism of action: immune activation
	Mechanism of action: e.g., immunosuppressant		

Risk-Based Approach to ADA Testing Strategy

The ADA testing strategy should be based on an immunogenicity risk assessment. Appropriately designed, validated, and executed immunogenicity assays and testing schemes provide the data that make risk assessments possible and predict the eventual outcome for patients. The frequency of sampling, neutralizing activity assessments, and qualitative or quasi-quantitative measurements may all depend on perceived risk.

In clinical studies, patient safety is of primary concern, and the extent of ADA characterization necessary depends on the potential risk of ADA-related sequelae. The type of drug should also be taken into account. For example, for a multicomponent fusion protein that contains at least one component with a potentially high risk of adverse events, a domain-mapping method (i.e., reactivity of ADA with individual components) is recommended.

Generally, more extensive ADA testing and characterization should be applied if the risk of clinical adverse effects is high. Samples should be analyzed and characterized based on the timing and incidence of the ADA response as well as the occurrence and severity of clinical side effects. A higher risk of ADA incidence normally does not warrant extensive characterization of ADA, and usually the risk of clinical consequences drives the bioanalytical strategy. Nevertheless, some investigations may be driven by the need to understand the cause of a high ADA incidence (e.g., the reactivity of ADA with aggregated versus nonaggregated drug).

The following step-wise, risk-based testing strategy can be refined depending on the product's level of risk and during the design of the clinical studies to ensure that the maximum amount of data can be gained, including correlations to PK, PD, etc.

STEP 1

Develop ADA methods that are fit for purpose and are consistent with current industry best practices and regulatory guidance. Incorporate baseline and postdose ADA testing into the clinical study design, together with concurrent testing for PK plus any relevant PD, safety, or efficacy markers that will facilitate interpretation of ADA data. The analysis of results from ADA testing should be built into study analysis plans.

STEP 2

Test all pre- and post-dosing samples for ADA. Two important tests that should be carried out in all cases are the ADA screening assay and the drug inhibition or immunoglobulin depletion confirmation assay. Report as negative any ADA results below the assay cut-point and with drug levels below the interference levels, as well as those that test negative in the confirmatory assay. Test methods that are capable of sensitive ADA detection despite the presence of trough levels of drug are desirable. In their absence, samples containing drugs above the interference levels should be reported as inconclusive with a

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statement of possible drug interference. In such cases, ADA analysis could be performed later following a drug washout period to reach a conclusive result (refer to the section *Relative Sensitivity* of this chapter for further information). For the confirmed positive samples, ADA levels should be estimated, preferably by titration, but they can be reported in terms of relative mass units. Certain mass-based technology platforms may necessitate the use of relative mass units.

STEP 3

Samples deemed positive in *Step 2* should be tested for neutralizing ability and potentially other characteristics, depending on the risk assessment. In high-risk situations, NAb activity should be measured, typically using a cell-based assay. Depending on the drug's mechanism of action, sometimes a ligand-binding NAb assay format can be used if it is adequately proven to specifically detect NAb. Concurrently generated PK/PD/safety or biomarker data should be used to help interpret the clinical relevance of neutralizing antibody activity. In addition, determination of ADA isotypes and affinity may be helpful in the overall assessment of the immune response. Allergic reactions associated with drug administration may necessitate measurement of drug-specific IgE, although detection may depend on the sampling scheme and method design.

DESIGN OF IMMUNOASSAY-BASED TEST METHODS

Immunoassay methods for ADA detection generally are complex and require a broad understanding of multiple technical challenges. Screening assays that serve as the key first step in the immunogenicity testing scheme are designed to have a certain false positive (rather than false negative) rate in order to maximize the sensitivity for detecting ADA. In addition, using a risk-based approach, it is more appropriate to have 5% false positives rather than false negatives during this initial screening phase. Typically, positive screening samples are confirmed to contain drug-specific ADA in confirmation assays before the determination of the level of ADA (titers) or any additional characterization.

Screening Assays

In their simplest form, screening ADA assays immobilize the therapeutic protein on a microtiter plate or onto beads to capture ADA (solid phase) or co-incubate a labeled therapeutic protein at a predetermined concentration with the sample containing ADA (solution assay). The bound polyclonal ADA is then detected using a labeled secondary reagent or labeled drug. Limiting the number of wash steps or reducing wash fluid dispensing rates may increase the detection of antibodies with fast off-rates on assay platforms that use wash steps. Generally, screening assays are designed to detect classes of antibodies that may be most relevant to the product's route of administration, e.g., IgA for mucosal routes of administration. Although the most common ADA raised against protein therapeutics are of the IgM and IgG isotypes, other isotypes of ADA including IgE and IgA may require detection based on the clinical response and the route of product administration. In addition, depending on how rapidly ADA responses develop and the half-life of the therapeutic, it may be feasible to detect the development of ADA initially of the IgM isotype that later affinity matures to an IgG isotype following repeat administration of product.

Because screening assays serve as the first step in the immunogenicity testing program, these assays generally are configured to have moderate throughput and often are automated. The various technology platforms used to develop screening immunogenicity assays have inherent strengths and weaknesses as outlined in *Table 3*. Development of a bioanalytical strategy to use a certain technology platform for assay development should take into consideration the nature of the product (e.g., a therapeutic protein or a monoclonal antibody), potential sources of interference in the assay (e.g., therapeutic concentration anticipated in patient samples, soluble target based on co-medications, and biology), and disease-specific interfering or cross-reacting factors (e.g., rheumatoid factor).

Table 3. Advantages and Disadvantages of Various Assay Types Currently Used to Assess ADA

Assay Type	Advantages	Disadvantages
Direct/Indirect ELISA	High throughput	Potential for high background
	Readily available	May not be specific
	Easy to automate	Utility depends on capability to detect different Ig subtypes
	Inexpensive	Drug tolerance lower for solid-phase ELISAs
	Readouts can increase sensitivity (e.g., electrochemiluminescence)	Excessive washes can decrease detection of low-affinity ADA
	Drug tolerance higher for solution-phase ELISAs	

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Table 3. Advantages and Disadvantages of Various Assay Types Currently Used to Assess ADA *(continued)*

Assay Type	Advantages	Disadvantages
Bridging Format	Low background	Difficult to confirm presence of IgM
	Highly specific	Need to label product
	Readily available	Reduced ability to detect low-affinity antibodies and IgG_4 because of single-arm binding between bound ligand and detector
	Easy to automate	
	Inexpensive	
	Format can be used across species and detects all isotypes	
	Can be used across detection platforms (colorimetric, electrochemiluminescence, etc.)	
Surface Plasmon Resonance/Biolayer Interferometry Assays	Flexibility to characterize immune response (concentration and relative affinity)	Expensive technology
	Higher drug tolerance and can detect low-affinity antibodies	Limited suppliers
		Moderate throughput
Radioimmunoprecipitation Assays	Inexpensive	Radioactive waste
	Highly sensitive	Need to frequently requalify radiolabeled reagents because of short half-life
	Better for high-affinity antibodies	Utility depends on capability to precipitate all relevant antibody present
		Can be less useful for low-affinity antibodies

In addition, analysts should consider whether the method does not result in an underestimation of ADA-positive samples because the ADA binding epitopes on the capture reagent are blocked with a tag or by coating on plates or beads. Another point for consideration in the design and development of an ADA assay is the adaptability of the test for both nonclinical and clinical matrices. Although most assay formats can transfer readily from nonclinical to clinical use, there are exceptions. Further, clinical ADA assays should be qualified or validated for use with samples collected from a similar patient population. Here again, although most ADA screening assays may not show unique matrix interferences between one disease matrix and another, there may be exceptions. Immunoassays used for ADA detection generally are quasi-quantitative methods because standardized, species-specific (especially human) polyclonal ADA calibrators generally are not available. The positive controls, typically developed in-house as hyperimmune serum in animals or by phage display serve as surrogates for the drug-induced ADA in treated patients.

As depicted in *Table 3*, some of the more common assay formats currently in use for development of screening assays include plate-based or bead-based enzyme-linked immunosorbent assays (ELISA; see also USP general chapter *Immunological Test Methods—Enzyme Linked Immunosorbent Assays (ELISA)* ⟨1103⟩) with colorimetric, fluorometric, or luminescent read-outs, plate-based or solution-phase electrochemiluminescent (ECL) or ELISA assays, surface plasmon resonance assays (SPR; see also USP general chapter *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩), or biolayer interferometry assays, and radioimmunoprecipitation assays (RIPA). In order to differentiate positive from negative responses, assay cut-points are statistically determined using samples collected from the target population. The assay cut-point also helps to determine the assay sensitivity. An incorrectly established cut-point may result in false negatives or too many false positive responses that should be ruled out as drug-specific responses in the confirmation assay. Assay performance typically is optimized during development by evaluation of the following parameters: sensitivity (lowest amount of detectable antibody in a sample demonstrated using surrogate controls); specificity (likely detection of a true positive rather than a nonspecific interaction); precision (reproducibility of results from multiple analyses); interference (interfering substances in sample matrix, including the administered drug, that affects assay sensitivity); and stability and robustness (likelihood of optimal assay performance over time). After optimizing these parameters, analysts typically validate the method for its intended use. If the initial assay cannot meet the performance goals (e.g., because of poor sensitivity or high backgrounds), then analysts should either improve and validate the first assay format again or develop and validate more than one assay format.

Confirmatory Assays

Samples that are positive in the screening assay usually are confirmed in a second assay that includes adding a certain fold excess of the therapeutic. This is intended to demonstrate that a positive signal seen in the ADA screening assay is caused by the presence of drug-specific antibodies. Because the cut-point for the screening assay is set to result in the detection of approximately 5% false positives, the confirmatory assay is used to rule out the false positive samples from further analysis. Multiple options are available for performing confirmatory assays. Usually a soluble drug is added to the sample and should compete with the plate-bound immobilized drug for binding to sample ADA. A specific interaction to a soluble drug results in a decrease in the assay signal. As with the screening assay, the cut-point for the confirmation assay is established statistically. Verification of the presence of drug-specific antibody can also be performed using an orthogonal method on a different assay platform that may have different nonspecific binding profiles. Analysts should take care while adopting this approach to ensure an adequately sensitive assay in order to avoid a false negative reaction. Finally, the specificity of ADA to a drug can also be confirmed by depleting all immunoglobulin from a sample (e.g., using a Protein A or G column) followed by reanalysis of the depleted sample. In the latter approach, the depleted sample scores negative in the assay if an antibody caused the original

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signal. Validation of the confirmatory assays helps ensure that the results from the confirmatory assay are appropriately interpreted.

Characterization Assays

Following screening and confirmation, the relative level of ADA in a positive sample is assessed by titration. The most common approach is to serially dilute the sample and report the reciprocal of the highest dilution factor at which the sample tests positive, or the titer of the sample. The higher the sample dilution (and therefore the ADA titer value), the higher is the concentration of circulating ADA in that sample. This approach has been used historically to report serological data from vaccine studies. Another less frequently used approach is to express the amount of ADA in the sample in mass units relative to a surrogate standard. Although this approach has the advantage of relating the amount of antibody in the sample to assay sensitivity, analysts should recognize that the calibrator may not be representative of the polyclonal ADA responses under measurement.

Traditional approaches to dilutional linearity testing do not apply to ADA assays. However, when analysts express ADA data in terms of titer values, they also should demonstrate that the positive control(s) displays reasonable (relative) linearity of dilution.

In addition to performing titration, analysts routinely characterize positive ADA samples in neutralization assays to determine the in vitro effect of ADA that might reflect the in vivo biological or pharmacological activity of the therapeutic product. Additionally, the isotype(s) of the ADAs also can be analyzed. Isotype(s) identification is sometimes performed in a multiplexed manner. Using one fluorescent multiplex platform, analysts mix each sample with multiple secondary reagents that are specific to different immunoglobulin isotypes and are labeled with unique fluorochrome labels.

An SPR-based platform also can be used for this purpose (also see *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩). The isotype of an ADA can be determined by observing binding of isotype-specific reagents (such as an anti-human IgG_1 Ab) to the ADA that has been captured by immobilized drug. Analysts should take care when identifying and validating the isotype-specific reagents because unexpected cross-reactivity often is observed. Isotyping can help understand the maturation of the immune response. For example, an ADA response that is comprised solely of IgM antibodies is an immature immune response without T-cell involvement and may or may not progress further. In contrast, an immune response comprised of IgG_1 and IgG_4 antibodies represents a more mature response that has already engaged more components of the immune system. ADA titration and characterization assays are validated routinely using many of the same parameters as screening and confirmation assays to ensure consistent assay performance.

VALIDATION OF IMMUNOASSAYS

Method validation is a process of demonstrating, by the use of specific laboratory investigations, that the performance characteristics of an analytical method are suitable for its intended use (see also USP general chapter *Validation of Compendial Procedures* ⟨1225⟩). The level of method validation depends on the stage of product development and the risks associated with the product. A partial validation involving assessments of method sensitivity, specificity, and precision requirements with less emphasis on robustness, reproducibility, and stability may be adequate for the earlier stages of clinical development (Phase 1–Phase 2 studies), whereas fully validated methods are required for pivotal and postmarketing studies.

Validation of an assay before use of the method for sample bioanalysis is called *pre-study validation*, and amendments to this process may be made between studies. This process maps out the performance characteristics of the assay and should demonstrate that the method is suitable for its intended purpose when it is subsequently applied to study samples. In contrast, *in-study validation* refers to the monitoring of assay performance during study-phase applications of the assay in order to ensure that the assay remains valid and that the resulting bioanalytical data are reliable.

Reliable performance of the assay also depends on all the elements spanning bioanalytical testing and data manipulation, such as assay reagents, analysts, equipment, and computer programs. In essence, the assay is a system comprising several elements other than assay steps and reagents alone. Pre-study validation therefore establishes system suitability (establishment of criteria for control samples that are used to accept or reject runs and imprecision limits for individual samples), and then in-study validation continues to verify it. Critical changes to methods often require additional validation (partial or full), sometimes leading to the revision of the system suitability criteria.

Minimum Required Dilution

During assay development the minimum required dilution (MRD) can be defined as the dilution level of the ADA negative sample that results in the highest signal-to-variability ratio (or Z' factor).

The ability to dilute such samples also should be assessed to ensure that the chosen MRD is adequately distal to any prozone (hook) effects that may have been observed. Although they are rare, some unusual prozone effects may require the test method to include more than one dilution of a test sample to minimize false negative data.

The MRD should be evaluated during the assay development/design phase, i.e., before analysts initiate the validation experiments, so analysts do not need to repeat the evaluation during validation. It can be established using 10 individual drug-naïve ADA negative samples, each tested in 2-fold serial dilutions (e.g., a range of 1:5 to 1:80). The MRD is sometimes defined as the dilution level that results in the highest signal-to-background ratio when typically the background is the dilution matrix. However, this definition ignores the variability in the signal. Therefore it is preferable to define the signal-to-background value and include variability.

One way of doing this is to use a Z' factor that includes both the intensity of the assay readout and its variability at different dilutions (see the *Appendix* for more information). The Z' factor for each dilution level is obtained from the formula [(mean (S) – 3 SD (S)] – [mean (B) + 3 SD (B)]/[mean (S) – mean (B)], where S is the assay signal of the diluted sample and B is the background signal. Thus, the MRD is the dilution that results in a desired value for the Z' factor and signal-to-background ratio.

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This metric is widely used for high-throughput screening assays to ensure adequate confidence in the ability to differentiate between truly active versus inactive compounds. An inappropriately large MRD can compromise the sensitivity of an assay.

Pre-Study Validation

Pre-study validation establishes the following:

1. Assay cut-points
2. System suitability criteria
3. Relative sensitivity
4. Specificity
5. Selectivity/interference
6. Precision
7. Robustness
8. Reproducibility
9. Stability

Assay Cut-Points

Because of the quasi-quantitative nature of ADA detection methods, the use of a decision threshold or cut-point becomes necessary to discriminate between ADA-positive and -negative samples. Because the screening assay and specificity confirmation assay produce different types of results, separate cut-points are necessary. In some instances, a different cut-point also may be necessary for evaluating titers of confirmed positive samples from the titration assay. Some key points in the evaluation process are summarized briefly below (more information is available in the *Appendix*).

SAMPLES FOR CUT-POINT EVALUATION

Samples from an appropriate population should be used for the development of assay cut-points. In some cases it may not be practical or feasible to obtain matrix samples from a population that has a target disease before initiating pre-study validation experiments. Consequently, samples from healthy drug-naïve subjects are used commonly to establish the initial cut-points. This approach is preferred for a conventional Phase 1 study with normal volunteers. When the clinical program progresses beyond Phase 1 and samples from the target disease population become available, it is more appropriate to re-evaluate cut-point data for the target population. If the distribution of assay responses with respect to both the mean and variability are comparable between the target population and the normal volunteers, then the same cut-point can be used. If not, target disease-specific cut-points are more appropriate, and fixed or floating cut-points computed from the data obtained from the baseline samples from a clinical trial can be considered.

SCREENING CUT-POINTS

The screening assay cut-point is a signal in the screening assay that identifies a sample that is likely to contain ADA (termed a *screen positive* or *potential positive* sample) versus an ADA-negative sample. A screening assay cut-point is established during pre-study validation based on a systematic and statistically rigorous analysis of assay responses from a panel of individual samples that are considered to be representative of a drug-naïve target patient population.

To determine the screening method cut-points for clinical assays, analysts should use samples from at least 50 drug-naïve individuals for a robust evaluation. If additional indications are targeted, analysts should test at least 20 drug-naïve individuals per indication. If the variability is significantly different from the original indication, then additional drug-naïve individuals may need to be tested. If not, then the original cut-point can be applied to the additional indication. For nonclinical assays a total of at least 25 drug-naïve individuals should suffice. To ensure this cut-point is robust, at least two analysts should perform this experiment over three days in at least two different plate layouts. A balanced experimental design and plate layouts will help avoid potential confounding between analysts, subject samples, run dates, etc. For clinical ADA assays, if multiple disease-state populations are being tested they should be distributed evenly across the plates to ensure they are properly balanced across plates and runs. Statistical outliers of the sample results should be examined and eliminated, e.g., using outlier box plots defined in terms of quartiles and interquartile range. In addition, confirmed reactive samples (e.g., via immunodepletion) can be excluded as well.

Three types of screening cut-points can be calculated for application during the study phase—fixed, floating, and dynamic—and one of these should be appropriately chosen for study phase bioanalysis.

Fixed cut-point—A fixed cut-point is a cut-point that is determined in pre-study validation and subsequently is used for the in-study phase. The fixed cut-point is used for analyses of test samples until there is a need to revalidate or change the cut-point (e.g., because of a critical change in the assay, assay transfer to another laboratory, upgraded instruments, etc.). The cut-point value can be fixed within a given study, for a target population, or across studies and multiple target populations. In order to use this approach, one should statistically demonstrate similar means and variances across runs during pre-study validation. A fixed cut-point can be determined based on the mean + 1.645 SD (standard deviation), which represents the 95th percentile of the population under normal distribution (and therefore is expected to identify approximately 5% of the samples as false positives). The standard deviation should include different sources of variation such as the intra-run, inter-run, inter-analyst, and inter-subject variability. If the data are not normally distributed, appropriate transformations (typically log transformations) can be used. If transformation doesn't help, usually it is acceptable to determine the nonparametric 95th percentile. However, in preclinical trials it may be considered adequate to use a cut-point at the 99th or 99.9th percentile because immunogenicity of a protein normally results in high antibody titers. Alternatively, even if the validation data suggest similar means and variances

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across runs, to account for possible deviations between assay runs during the in-study bioanalysis phase, it would be safer to apply a floating cut-point.

Floating cut-point—A floating cut-point is a cut-point calculated by applying an additive or multiplicative normalization factor, determined from the pre-study validation data, to the biological background obtained during the in-study phase (see Appendix G of Shanker et al., 2008, in the *Appendix* for details). The biological background may be represented by the negative control (pool of matrix from subjects that are negative for ADA), the assay diluent, or the predose subject sample (subject-specific cut-point). The method for determining floating cut-point uses the variation estimate from the pre-study validation that includes different sources of variation such as the intra-run, inter-run, inter-analyst, and inter-subject variability. Such a cut-point is recommended when the means of drug-naïve samples are not similar but the variances are similar across runs. When a negative control is used for normalization, analysts also should ensure that the negative control results represent the drug-naïve matrix sample results of the target population. This is accomplished by demonstrating that the signal of the negative control trends directionally with the signal of the individual samples. Alternatively, the use of assay diluent for normalization or pretreatment subject ("baseline") sample results may be more appropriate. However, a pre- versus post-dose ratio might be a better solution.

Dynamic cut-point—A dynamic cut-point is a cut-point that changes between the plates in a run, between runs in a study, or between studies, and it does not apply the variation estimates from pre-study validation. The latter characteristic differentiates it from a floating cut-point. This approach is necessary only where means and variances significantly differ between runs. Because this approach entails testing of several individual drug-naïve samples for the evaluation of a run-specific cut-point, it consumes a large portion of the plates from each in-study run and therefore is not practically feasible, especially when analysts use 96-well plates instead of 384-well plates. Differences in variability between assay runs sometimes can be resolved by further optimization of some key steps in the assay protocol or by resolving some analytical issues. In some cases, the differences in variability can be attributed to different analysts or instruments, and use of an analyst-specific or instrument-specific floating cut-point may resolve this issue. If further optimization does not resolve the situation or if the causes are not clear, another practical alternative may be to pool the variability across all runs and use this pooled variance for floating cut-point evaluation during the in-study phase.

SPECIFICITY CONFIRMATION METHOD CUT-POINT

Because of the conservative approach of incorporating a 5% false-positive rate into the computation of the screening cut-point, the elimination of false-positive samples via confirmation of drug-specific binding is an important component of ADA bioanalysis. It is also important to understand the level, if any, of the drug itself within the sample.

The amount of change in assay signal that differentiates drug-specific binding from nonspecific binding is referred to as the *specificity cut-point*. The specificity cut-point should be determined by an objective approach in the context of assay variability near the low positive range of the assay. To determine the specificity cut-point, drug-naïve negative samples from at least 25 individuals should be evaluated (however, more are commonly used when available), with and without drug preincubation. Ideally these samples should be the same as those tested during the screening cut-point evaluation, and the unspiked and spiked counterparts of the individual subject samples should be tested together in the same plates.

The mean percent change from the unspiked sample (inhibition) and SD are calculated. The mean inhibition plus 3.09 SD (if a 0.1% false positive rate is desired) represents the specificity cut-point. As in determination of the screening cut-point, specifically reactive samples after preincubation with drug (i.e., those that contain pre-existing antibodies) and statistical outliers should be eliminated in order to make the specificity cut-point more conservative. The analytical process outlined above for the screening cut-point applies for the evaluation of the specificity cut-point as well.

Alternative approaches such as the use of *mock low positive* samples in which the individual drug-naïve samples are spiked with a low concentration of a positive control sometimes is considered for this cut-point evaluation. However this method is subjective and is not recommended because it depends on the concentration of the positive control and the unique affinity/avidity of the positive control that may or may not represent true positive patient samples. Additional sources of information regarding the relative statistical merits of these approaches and methods for verifying the assumptions are listed in the *Appendix*.

TITRATION METHOD CUT-POINT

The titration method cut-point is a test result value below which further serial dilution of an ADA-positive sample produces negative assay results. Typically, the screening assay cut-point is used as the titration cut-point, but the validation of a separate titration method cut-point can become necessary when the signal from the assay diluent or matrix causes higher results than the screening assay cut-point (because of a blocking effect of serum) or if samples at a dilution higher than the MRD do not generate consistently negative results, i.e., when the screening cut-point falls on the lower plateau of the positive-control dilution curve. In such instances, the same data generated during a screening cut-point experiment can be used to define the titration cut-point using a 0.1% false positive rate threshold criterion (i.e., Mean + 3.09 SD). During bioanalysis, confirmed positive patient samples that fall between the screening cut-point and titration cut-point can be assigned a titer value equal to that of the MRD.

CROSS-REACTIVITY METHOD CUT-POINT

If applicable, ADA-positive samples that are confirmed to be specific to the drug can be further characterized for cross-reactivity to other related antigens. Like the specificity confirmation assay, a cross-reactivity test method may require a preincubation step with and without the related antigen. Cross-reactivity to the antigen is confirmed when the percent inhibition of signal in the presence of the antigen is greater than or equal to the cross-reactivity method cut-point. The methods for determining the cross-reactivity method cut-point are similar to those for the specificity method cut-point, although it also may be acceptable to apply the drug specificity confirmation cut-point.

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Defining System Suitability

ASSAY CONTROLS

ADA-positive controls can comprise polyclonal or monoclonal anti-idiotypic antibodies. They should be affinity purified and quantitated to enable assay validation. Each run (or plate) should include at least a low level of positive control (*low positive control*) and a negative control, but the inclusion of a higher level control (*high positive control*) also can be useful in monitoring method performance. Tracking all of these controls over time can help ensure that the method is performing suitably. A low positive control helps ensure that the assay remains as sensitive during study phase bioanalysis as during the pre-study validation.

On the one hand, the low positive control should produce a response that can be seen reproducibly above the cut-point, but sometimes it may result in a signal that is below the cut-point (thereby failing or invalidating the assay). On the other hand, choosing an unreasonably high concentration for a low positive control may produce an assay signal that is substantially above the cut-point, which is inappropriate. To provide objectivity to the selection of a low positive control concentration, it is useful to think in terms of assay rejection rates, i.e., the percentage of assays (plates) that fail because the low positive control produces a result below the cut-point. As an example and in order to understand if the low positive control is sufficiently low, a 1% rejection rate may be a reasonable target for a low positive control. This is calculated as mean + $t_{0.01,df}$ × SD, where mean and SD are determined using the data from the sensitivity experiment or related assay development data, and $t_{0.01,df}$ is the critical value determined from the *t*-distribution corresponding to a 1% false positive rate and *df* is the degrees of freedom that depends on the number of samples and runs used in the calculation. This theoretically implies that about 99% of the data from the low positive controls will be at or above the cut-point.

An optional high positive control can be useful for methods that are prone to hook effects, tracking assay performance, reagent qualifications, and troubleshooting. The concentration of the high positive control should be chosen from the upper end of the linear range of the dilution curve, usually just below the upper plateau of the curve.

SYSTEM SUITABILITY CRITERIA

System suitability criteria using assay controls help ensure that an analytical procedure remains valid for use. Acceptance ranges (system suitability criteria) for quality controls should be established by statistical evaluation of the experimental data acquired during assay validation.

When the floating cut-point approach is deemed necessary and is used for the screening cut-point evaluation, the system suitability criteria or limits can be defined for the ratio of the low positive control to the negative control and for the ratio of the high positive control to the low positive control or a negative control instead of defining limits separately for each positive control. It is also useful to apply acceptance criteria for intra-assay precision (variability of signals of replicates in an assay) for the in-study phase. Although data from assays that fail acceptance criteria during the in-study phase should be rejected, setting criteria for passing or failing assays in pre-study validation experiments should be avoided because these potentially can lead to the exclusion of some validation data, resulting in an inaccurate estimate of analytical error. All assays during pre-study validation should be included, and the only exceptions should be those rejected for an assignable cause (e.g., technical error).

Relative Sensitivity

No ADA-positive control can be expected to represent the spectrum of humoral immune responses observed in individuals treated with study compounds. The sensitivity of ADA assays is highly dependent on the nature of the positive control reagent(s) so that high-affinity positive controls often produce better sensitivity values than lower affinity positive controls in the same assay. Analysts should consider this when they choose controls, as well as when they estimate assay sensitivity. Moreover, because the drug itself can interfere with ADA detection, the sensitivity of ADA detection becomes progressively worse in the presence of increasing concentrations of drug within the sample. Despite these caveats, the determination of assay sensitivity is valuable when analysts choose an optimal ADA detection method or platform, a low positive control for validation, or assess the suitability of an assay. The assessment of assay sensitivity in the presence of an interfering drug (drug tolerance) is critical for understanding the suitability of the method for detecting ADA in dosed patients. ADA assay sensitivity should be defined not as a single value, but as a set of at least two values: (1) the concentration of positive-control ADA detected within undiluted matrix in the absence of any drug and (2) the concentration of positive-control ADA detected within undiluted matrix in the presence of drug levels expected at the time points when samples for ADA analysis are taken. Assays should, in general, demonstrate a sensitivity of at least 500 ng/mL for methods applied to clinical studies (or 1000 ng/mL for nonclinical studies) to show suitability for intended purpose—that is, for the detection of clinically meaningful ADA, although assay sensitivity should be justified on a case-by-case basis. It is not useful to express sensitivity in terms of antiserum titers, and thus sensitivity should be assessed using monoclonal antibody or affinity-purified polyclonal preparations. Analysts can evaluate sensitivity by means of two assay runs performed by two independent operators (when feasible) for a total of at least three runs.

To assess sensitivity in the absence of a drug, analysts should prepare mock samples with known concentrations of ADA that are serially diluted (usually 2- to 3-fold serial dilutions) in matrix pooled from drug-naïve individuals and evaluated according to the screening method until the assay results of the dilutions in matrix are below the screening assay cut-point. The lowest concentration of ADA that is consistently found (for example, using a 95% upper confidence limit based on the number of runs or operators) above the screening assay cut-point is determined to be the sensitivity of the assay. Alternatively, it can be the lowest concentration of ADA that is found to be above the screening assay cut-point in all runs by all operators or in 19 of 20 runs (see the *Appendix* for more information).

To assess sensitivity in the presence of a drug, two alternative experimental approaches could be considered: (1) titrate the drug into undiluted matrix containing set concentrations of a positive-control ADA (e.g., 250, 500, or 750 ng/mL). Report the highest concentration of the drug at which ADA remains detectable. (2) Alternatively, because immunogenicity samples often

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are taken at drug trough time points, prior knowledge of the anticipated trough drug concentration range could be used to determine the assay sensitivity in the presence of the expected concentrations of the drug.

Specificity

Specificity refers to the ability of a method to detect ADA that specifically binds the drug molecule, its domains, or components. The assay is developed and optimized based on the ability of the positive-control ADA to specifically bind the drug. During validation, results of the specificity confirmation assay support assay specificity.

Selectivity and Interference

The selectivity of an ADA assay is its ability to identify a positive control in biological matrix samples that may contain potential interfering substances and is an important concern for ADA detection assays. Such matrix effects typically arise from nonspecific binding interactions between a matrix-based factor and the ADA or from specific binding of unknown factors. During validation, analysts assess the selectivity of the ADA assay by looking at the recovery of analyte (represented by a positive control sample) from matrix samples that contain the potential interferent(s). One caveat here is that the selectivity of an ADA assay, as assessed using the positive control, may not reflect the selectivity of the assay when it is used with actual nonclinical or clinical samples.

Interference is the property of a factor (most commonly the drug itself and its target, if soluble) to affect assay results positively or negatively. It should be evaluated using a low positive ADA test sample that is spiked into a sample matrix from drug-naïve patients. Each potential interfering factor should be tested at a physiologically or pharmacologically relevant range of concentrations. The highest concentration of the interfering factor that does not alter the classification of the test sample (e.g., an ADA sample that remains positive relative to the screening assay cut-point) is defined as the tolerance of the assay to that interfering factor. For therapeutics that have a long terminal half-life, the main interferent in an ADA assay is the drug itself. As discussed previously, the drug tolerance of an assay should be interpreted as the sensitivity of the method in the presence of interfering drug.

Other endogenous interferents include oligomeric drug targets, or the target's soluble receptor may interfere with ADA detection. In addition, certain sample pretreatments performed to reduce drug interference can release drug target from drug-target complexes, leading to subsequent interference problems. Hence, analysts should carefully evaluate pretreatment steps such as acid dissociation during assay development to mitigate the risk of inaccurate data.

Precision

Precision is a measure of the variability in a series of measurements for the same material run in a method. The acceptance criteria for the precision of ADA assays should be within the range commonly expected for immunoassays. These criteria also should be appropriate for the assay platform and should be fit for purpose. During assay validation, precision should be determined in experiments that are run at the level of intended use during the study phase (e.g., number of plates, samples per plate, etc.).

The acceptance criteria for precision should be within the range commonly expected for immunoassays. These criteria also should be appropriate for the platform used, guided by assay development data and experience with the technology platform and assay method. Additional information is found in the *Appendix*.

SCREENING AND CONFIRMATORY METHOD PRECISION

For ADA screening assays with numeric readouts (as opposed to categorical yes/no readouts), assay precision can be determined using data from at least six independent assay runs of the assay positive controls (low positive and high positive controls). Typically, estimates of intra-assay precision (interreplicate variability, also called intra-assay repeatability) and interassay precision (also called interassay repeatability, or intermediate, total, or overall precision) are reported as percent coefficient of variation (%CV).

Intra-assay precision (repeatability) is the variability of assay results when the same material is tested multiple times within the same run. Interassay precision (also termed intermediate or total precision) is the variability of assay results when the same sample is tested in separate runs, over separate days, and by multiple operators (or only one operator if the study phase bioanalysis is intended to be performed by only one operator). These are expressed as %CV of ADA signals. Data from the replicates of negative and positive controls from each of all the runs tested during the pre-study validation phase are pooled and analyzed within the framework of random-effects ANOVA, resulting in estimates of intra-assay %CV and interassay %CV. Analysts should consider positional effects by varying sample position on microtiter plates because these effects (e.g., edge effects) can influence the assay precision. One should use the same number of test and control sample replicates during validation as are used in the assay during routine use.

Similarly, intra- and inter-assay precision estimates for the confirmatory assay can be derived using the percent inhibition data of the spiked versus unspiked low positive control samples from multiple assay runs (at least six) in the pre-study validation.

TITRATION ASSAY PRECISION

In order to determine the precision of a titer, two or more analysts should assay serial two-fold dilutions of five or more mock high positive control samples in at least six runs. Mock high positive control samples can be obtained by spiking individual negative sera from the target population with a high positive sample. The titer then is determined by interpolation of each of the dilution curves, and the overall mean and SD are calculated. Then intra- and inter-assay precision (%CV) can be determined.

A recommended but more rigorous approach is to use these data to define a minimum significant ratio (MSR): $MSR = 10^{t \cdot sqrt(2) \cdot SD}$, where SD is the overall standard deviation (intra-run plus the interrun variation) of the titers in common (base 10) log scale,

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and t is the threshold from Student's t-distribution with $n - 1$ degrees of freedom (n = number of runs). The calculated MSR reflects the smallest fold-change in the titer values that can be considered as statistically significant ($P < 0.05$)—i.e., if MSR = 5, then titers that are different by more than five-fold can be considered significant. In addition to serving as an indicator of the level of variability in the titers of the positive control, this MSR evaluation also can be an approximate criterion for comparing samples with confirmed pre-existing antibodies in baseline versus posttreatment samples in order to assess treatment-induced immunogenicity. The MSR applies only if the titer is interpolated and does not apply to endpoint titers.

Robustness

Robustness is an indication of the reliability of an assay. It is assessed by the capacity of the assay to remain unaffected by small but deliberate variations in method performance that would be expected under relevant, real-life changes in standard laboratory situations. The choice of robustness variables to test during validation should be based on the knowledge of the assay and its associated risks. Some common variables are microtiter plate lots, incubation times, temperature, and reagent lot and concentrations. Study samples or positive control samples can be used to test assay robustness. The use of acceptance criteria for system suitability controls during robustness validation (computed from the assay development and optimization data or validated system suitability control acceptance criteria) is recommended. Continuous monitoring of an assay during validation and beyond with strict records of key assay parameters (e.g., incubation times, pipetted volumes of critical reagents, operators, etc.) may help identify some of the robustness factor interactions if sufficient data are accumulated.

Reproducibility

Assay reproducibility according to USP general chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1) *Validation of Analytical Procedures: Text and Methodology* is the reliability of a method when performed in two or more laboratories. In the context of method transfers and interlaboratory method validity demonstrations, assay reproducibility is the same as a cross-validation.

Reproducibility is applicable only if an assay will be run by two or more independent laboratories during in-study phase bioanalysis. Reproducibility is an assessment of the transferability of an assay, i.e., the validity of testing samples in two or more laboratories and the comparability of data produced by them. Reproducibility assessments do not consider routine changes in an assay such as interequipment or interanalyst imprecision. Such contributors to variability (often referred to as intermediate precision factors) are part of the reproducibility variability.

Study phase acceptance criteria for system suitability controls are established in the originator laboratory (see below) during the original assay validation process. The performance of these controls can be compared across multiple laboratories. When only a single laboratory performs the ADA assay, however, reproducibility need not be validated until the method will be transferred to another laboratory.

Stability

It is useful to understand the optimal storage conditions for assay samples, controls, materials, and reagents, and they should be investigated as part of assay optimization before validation. Later, during assay validation, stability studies should evaluate assay performance following intended storage conditions. Ideally, stability testing conditions should mimic the expected sample, material, and reagent handling conditions, storage temperature(s), and varying lengths of storage time.

MATERIAL AND REAGENT STABILITY

ADA assays are stability indicating with respect to the applicable materials and reagents, and thus separate tests for reagent stability usually are not required for assay validation. During study phase bioanalysis, assay materials and reagents are presumed to be stable if the system suitability controls meet validated acceptance criteria. However, analysts should validate the stability of plates that have been prepared in advance (e.g., coated with capture antibody and blocked) and stored.

SAMPLE HANDLING AND STABILITY

ADA samples typically are collected in a serum or plasma matrix. For samples stored at or below –20°C, the stability of ADA are universally accepted, so this sample storage condition may not require validation. It is generally accepted that an ADA sample in serum or plasma will be stable after three freeze–thaw cycles and up to 2 years when stored at –70°C.

Documentation of Pre-Study Validation

Typically three types of assay-specific documents are created during pre-study validation: an assay validation plan or protocol, an assay method description, and an assay validation report.

An assay validation plan or protocol is recommended before analysts initiate pre-study validation experiments. This document should state the intended purpose of the method, a detailed description of the immunoassay and reagents or materials, a summary of the performance characteristics that will be validated, and a priori acceptance criteria for precision, robustness, stability, and, when appropriate, reproducibility. Some experimental detail and data-handling procedures should be presented in the validation plan because these details provide a clear guidance to the validation analysts and ensure better control over the resulting data.

A method description typically is established after pre-study validation but before the study. This provides a detailed description of the reagents, controls, and equipment needed to run the assay, together with a step-by-step operating procedure

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and information about processes for data reduction and interpretation. The point at which such a description becomes a Standard Operating Procedure (SOP) is specific to each manufacturer's quality system.

When validation is completed, manufacturers generally conduct a technical peer review of validation data, followed by a validation data audit. An assay validation report is created after the validation work is completed. This documents all of the study validation data, together with information about the methods and batches of reagents that were used. An audited report is approved by management and then is archived.

In-Study Validation

In-study validation (monitoring the maintenance of system suitability) and revalidation are critical components of any bioanalytical method. Hence, the validation of a method actually does not end until the method is retired from analytical use.

For in-study performance of quantitative bioanalytical methods, acceptance criteria for precision and accuracy generally are required. Because accuracy is *not* applicable for ADA methods, monitoring the performance of quality control samples reassures analysts that the assay system is suitable for its intended use, i.e., that the assay remains valid and is performing as well as it did during pre-study validation.

The use of a low positive control ensures the assay remains sensitive. Generally during study sample analysis the intra-assay (interreplicate) precision of the results of positive controls, as well as test samples (with assay signal at or higher than the screening cut-point), are controlled using system suitability acceptance criteria to ensure that meaningful data are consistently obtained. Results below the cut-point, however, may not be required to meet CV limit criteria.

LIFE CYCLE MANAGEMENT

Management of the performance of immunogenicity assays from initial clinical development through subsequent product life cycle requires a comprehensive understanding of the strengths, weaknesses, and capabilities of the method format, as well as of the critical assay reagents and assay performance characteristics. In addition, a well-defined plan for critical reagent production, characterization and qualification, qualification of suppliers of critical reagents, and characterization and qualification parameters for reagents produced in-house (aggregate level and labeling efficiency) help manage the risk of maintaining the assay and transferring the method to other laboratories.

When there are changes in critical method components, equipment, or the population that is studied with a particular ADA assay, an assay revalidation may be required. The revalidation may cover some or all validation characteristics (i.e., it may be a partial or whole assay revalidation). Use of lots or batches of assay critical reagents that are different from those used in pre-study validation do not require assay revalidation, but they must be supported by appropriate experimental qualification data for the new reagent to ensure maintenance of system suitability.

Another critical aspect of life cycle management is the development of a strategy to bridge clinical data between an existing and an improved assay format. Such changes typically occur in a product's life cycle because of postmarketing commitments or other needs. To facilitate comparison and cross-validation of the existing method to the revised versions, analysts should retain sufficient aliquots of the original lots of critical assay reagents. In addition, archiving of analyte-spiked samples as well as blinded patient samples is useful to bridge between reagent lots and methods in order to minimize drift in assay performance. Analysts should develop a written plan outlining the sort of changes to the existing assay or critical reagents that will warrant an assay qualification versus a cross-validation or full validation. A quality management document should include details such as the number of assays that must be performed, the number of analysts that will be used, required training for analysts, acceptance criteria to demonstrate equivalence between existing and revised methods, data analysis, and reporting method. This information demonstrates the robustness and consistency of the assay following changes. Quality controls that ensure assay equivalence include %CV, tolerance limits, EC_{50} values of slope, titer level, and signal-to-noise ratio. One approach commonly used to demonstrate equivalence of two immunogenicity methods is the demonstration of ≥90% concordance in archived sample results between the existing and revised methods.

Analysts should use archived samples with a range of positive values as well as an appropriate number of negatives to verify that a new assay segregates samples into positive and negative categories in the same manner as an existing one.

Another important consideration for life cycle management of critical assay reagents is the monitoring of long-term reagent stability under different storage conditions. A detailed stability testing plan includes storage temperatures (4°C, –20°C, and –70°C), aliquot volume, freeze–thaw cycles, and acceptable performance characteristics for assay qualification, and results should be documented. In this context, it may be prudent to archive patient samples to demonstrate the long-term stability of the polyclonal ADA response in actual patient samples.

APPENDICES

Appendix 1: Nonclinical Immunogenicity Testing

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Appendix 3: Design and Validation of Immunogenicity Assays

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Appendix 5: Regulatory Guidances for Clinical Immunogenicity Studies

FDA. Draft guidance for industry: assay development for immunogenicity testing of therapeutic proteins. 2009. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf. Accessed 01 December 2011.
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⟨1106.1⟩ IMMUNOGENICITY ASSAYS—DESIGN AND VALIDATION OF ASSAYS TO DETECT ANTI-DRUG NEUTRALIZING ANTIBODY

INTRODUCTION AND SCOPE

Administration of natural source or recombinant biologic medicines may elicit some degree of immune response leading to development of anti-drug antibodies (ADAs) in treated subjects. Neutralizing antibodies (NAbs) are a subset of ADAs that affect the biological activity of the biologic drug product. For the purposes of this chapter, NAbs are defined by their ability to neutralize the biological activity of a therapeutic in an in vitro system. This chapter does not address antibodies that may impact drug clearance. [NOTE—Two helpful references on this topic can be found in the *Appendix.*] Further clinical studies would be needed to assess changes in biologic activity on therapeutic outcomes.

NAbs can alter the biological activity of the therapeutic molecule by binding to one or more epitopes that lie within its active site(s). In addition, NAbs can interfere with active sites through steric hindrance (i.e., binding to areas of the protein that are near the active site), or by allosteric interactions (i.e., binding to a site on the drug and inducing a change in conformation that can interfere with the drug's activity). Therefore, it is important to monitor the immunogenicity of biological therapeutics throughout the drug product development cycle by using sensitive and reliable methods that not only determine the presence or absence of ADAs but also characterize whether they have neutralizing capability. The objective of this general information chapter is to provide practical recommendations on best practices that may be used for risk-based design and validation of anti-drug NAb assays.

As described in *Immunogenicity Assays—Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* ⟨1106⟩, immunoassays (or ligand binding assays) are typically used to screen for the presence of ADAs. Several assay formats can be used to determine whether a detected ADA is also a NAb. The first assay format, defined here as a functional NAb assay (thus having an actual biological readout), is most commonly used and can take the form of either a cell-based functional assay or non-cell-based functional assay (e.g., a biochemical assay for an enzyme therapeutic). Another major group of assay formats is based on NAb-mediated inhibition of ligand binding between therapeutics and their targets. These assays can take the form of

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either cell-based binding assays (e.g., where a cell-surface target exists) or non-cell-based binding assays (e.g., where a purified soluble receptor or drug target is utilized).

The decision to use a cell-based assay versus a non-cell-based assay is dependent on factors that include the drug's mechanism of action (MoA) and immunogenicity risk assessment, as well as the availability of relevant and sensitive NAb assays (*Figure 1*). Another important consideration in selecting the assay format is the degree of risk to patient safety that NAb formation would pose; thus, for therapeutics where antibodies pose a high risk to patients, the assay format should be sufficiently sensitive for detecting clinically relevant NAbs. The speed at which results are required can have an influence on the type of assay selected if the cell-based assay requires extended time to execute. Some practical considerations for making this decision are presented in the section *Risk-Based Approach to Assessing Neutralizing Antibodies and Their Consequences.*

Detection of drug-neutralizing activity in such in vitro assays facilitates improved understanding of any observed clinical effects resulting from altered pharmacological activity. A thorough investigation of the presence of NAbs and their characterization in relation to clinical parameters such as pharmacokinetics (PK), pharmacodynamics (PD), efficacy, and safety is therefore strongly recommended. NAbs would have to be produced at a concentration that is biologically relevant such that the effect of the antibodies could be detected in an in vitro assay, and these antibodies could bind with sufficient affinity to remain tightly bound to the therapeutic. In contrast, a low-affinity-binding antibody that recognizes an active region of a therapeutic protein might not remain bound to the therapeutic, and thus would not readily mediate a biological effect. However, one cannot rule out the possibility of low-affinity antibodies having an effect in vivo, and thus NAb assays should be designed to detect the widest range of antibody affinities that is practical (e.g., limit the number of wash steps).

Although the principles described in this chapter are generally applicable to most commonly used cell-based and non-cell-based NAb assays, modified approaches to assay design and validation may be required for certain products (e.g., enzymes), clinical uses (e.g., different dosing regimens or routes of administration), or patient populations. [NOTE—A list of helpful regulatory guidances and white papers is contained in the *Appendix*.]

FACTORS THAT INFLUENCE THE DEVELOPMENT OF NEUTRALIZING ANTIBODIES

Although it is not entirely clear why biological therapeutics induce NAbs only in some individuals, or why certain therapeutics induce more NAbs than others, research has shown that certain factors are typically associated with the generation of NAbs. The initial low-affinity response to a therapeutic protein (primarily composed of IgM antibodies) is rarely able to effectively neutralize the biological effect of the therapeutic protein. However, antibody response to a therapeutic protein can mature, usually upon repeated exposure. As the immune response matures, more epitopes of the therapeutic protein may be recognized by ADAs leading to an increased possibility of NAbs, as epitopes within the active region of the therapeutic protein are now recognized.

Ultimately, those factors that trigger development of a robust immune response require the trigger of T-cell mediated immune responses. Some of these factors include the presence of multiple T-cell epitopes (linear, 7–9 amino acid sequence recognized in the context of the major histocompatability complex class II or MHC II); specific product attributes, such as aggregation, that would encourage uptake into antigen-presenting cells; genetic sequence differences between the therapeutic protein and endogenous counterparts; degree of immunologic tolerance; and the amount, route, and schedule of the administration of the protein therapeutic. There is also a potential for residual host cell proteins to act as adjuvants. Therapeutic proteins that are very close in amino acid sequence to endogenous proteins are less likely to trigger a robust immune response. When the sequence is identical and the endogenous counterpart is fully exposed to the immune system, a high level of tolerance to the therapeutic protein is anticipated. In subjects with autoimmune disorders, this may not be the case, as tolerance to self-proteins has already been compromised.

NAbs are always defined within the context of the assay that identifies them. Thus, it is possible that not all subjects with NAbs are identified in any program. Also, it is important to recognize that if the sensitivity of a NAb assay improves, then the number of subjects with NAbs that are detected may increase. If the incidence of NAb-positive subjects increases, it is important to determine whether the change is related to assay performance or to an alteration in the product quality attributes of the therapeutic protein. NAb specificity is also important. For assays with relatively poor specificity, baseline (pretreatment) samples may show positive responses; however, such a response may not be due to antibodies directed specifically to the therapeutic under study.

DETERMINATION OF PRECLINICAL AND CLINICAL IMMUNOGENICITY

Nonclinical: Relevance and Scope of Preclinical Immunogenicity

Preclinical toxicology studies are generally used to suggest dosimetry and to define safety margins by evaluating idiosyncratic toxicity and specific target-organ toxicity caused by therapeutics. The primary requirement for these studies is to demonstrate that the animals are exposed to the therapeutic at the intended dosage levels throughout the study. As stated in the ICH S6 Guidance, this can be accomplished by analyzing the circulating drug concentration (PK data) in combination with data on PD markers, and/or by using immunogenicity assessments to facilitate interpretation of the exposure-to-toxicity correlation. As discussed in ⟨1106⟩, animal test systems have significant limitations in their ability to support specific conclusions on the potential for clinical immunogenicity, especially as it relates to MHC restriction, T-dependent B-cell responses, and the potential to neutralize the pharmacological effect of the therapeutic. However, evaluation of immunogenicity in nonclinical studies may be useful when there is substantial homology of the therapeutic to an endogenous counterpart in animals [e.g., erythropoietin (EPO) and thrombopoietin (TPO)]. Severe thrombocytopenia was reported in several animal species after dosing with autologous TPO molecules, due to development of circulating anti-TPO NAbs. In these studies, NAbs directly affected the pharmacological activity of both the endogenous protein and the exogenously administered therapeutic, making it difficult to interpret the toxicology study findings.

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NAbs are usually directly detected and characterized by in vitro assays. However, a decrease in the expression or activity level of a PD marker may be indicative of NAbs. If confirmation that the PD marker is due to the presence of NAb is desired then this can be confirmed with a NAb assay as a surrogate to infer the presence of NAbs. It is important to confirm that the change in the reduced PD marker is not merely due to clearance of drug from the circulation, an effect that can be caused by ADA, rather than due to "true" neutralization. As part of the immunogenicity evaluation, it also may be useful to characterize the immune-dominant epitopes for such high-risk proteins. Nonclinical sample collection and related considerations are discussed in ⟨1106⟩.

Although the measured incidence and titer of NAbs may not be the same in different animal species, induction of NAbs in multiple species may suggest a higher probability of NAb development in humans where significant homology exists within the biologically active site. Immunogenicity studies in transgenic animals expressing the gene encoding the protein of interest may be useful to help predict the relative potential of various forms of drug product to induce NAbs.

Clinical: Relevance and Scope of Immunogenicity Assessments

Evaluation of immunogenicity, including development of NAbs, remains an important part of the safety assessment in clinical drug development.

The scope of the immunogenicity assessment for therapeutics is based on the type of therapeutic, selected indication, pharmacology, route of administration, duration of treatment, and immune competence of the patient. The frequency of sample collection and analysis should be based on the timing and incidence of antibody responses as well as the occurrence and severity of clinical sequelae (see ⟨1106⟩ for more information). Because NAbs can trigger a range of possible clinical effects, specific and sensitive analytical methods are needed to (1) investigate the characteristics of antibodies induced over time (non-neutralizing and neutralizing), (2) reveal the duration of the ADA response (transient versus persistent), and (3) clarify the implications of the potential immunogenicity for clinical outcomes.

Adequate patient follow-up for measuring neutralizing ADAs should be implemented, because persistent NAbs generally develop later in the course of treatment and it is important to fully characterize the temporal nature of the antibody response. For example, careful follow-up could reveal a transient response in which antibodies appear, yet then disappear after a period of time such as 2–3 months. In addition to the scheduled, routine repetitive sampling, patients should be evaluated clinically in a symptom-driven manner whenever antibody development is suspected. Pre-approval clinical trials may fail to detect rare immunogenic events due to insufficient patient numbers for adequate statistical evaluation and/or an inadequate duration of drug exposure. Therefore, a postapproval immunogenicity surveillance program may be necessary for any therapeutic protein that carries a risk of generating clinically meaningful NAbs.

RISK-BASED APPROACH TO ASSESSING NEUTRALIZING ANTIBODIES AND THEIR CONSEQUENCES

Chapter ⟨1106⟩ describes in detail the principles of risk-based approaches to detection and characterization of antibodies directed against biologic drug products. The two primary concerns related to NAbs are (1) the therapeutic can lose pharmacological activity, which in turn can affect its efficacy, and (2) NAbs can cross-react with an endogenous, nonredundant protein. In addition, as with all ADAs, NAbs can contribute to other adverse effects such as the formation of immune complexes and potential deposition of these complexes in tissues and the vascular system. *Table 1* in ⟨1106⟩ summarizes important risk factors that may influence the severity of the clinical consequences from an ADA or NAb response. The basic risk assessment criteria outlined in *Table 1* in ⟨1106⟩ provide a useful orientation to this type of risk assessment. The various combinations of factors from the three risk categories (lower, medium, and higher risk), as well as the actual antibody properties, could result in a continuum of intermediate risks. For example, NAbs directed against recombinant human growth hormone can neutralize endogenous growth hormone, which is a vital, nonredundant factor. Yet decades of therapy with a number of different recombinant human growth hormone preparations have shown that attenuation of growth was not observed, even in the presence of the cross-reactive NAbs detected with an in vitro assay. Therefore, the actual risk associated with the recombinant human growth hormone could be viewed as intermediate between the high- and medium-risk categories, despite the existence of the endogenous counterpart.

Potential assay formats are selected based on MoA on a case-by-case basis with proper consultation with regulatory agencies when needed, while the analytical (or immunogenicity assessment) strategy is driven by the risk of immunogenicity for the specific therapeutic. *Figure 1* presents a flow chart with some useful options for NAb assay format selection.

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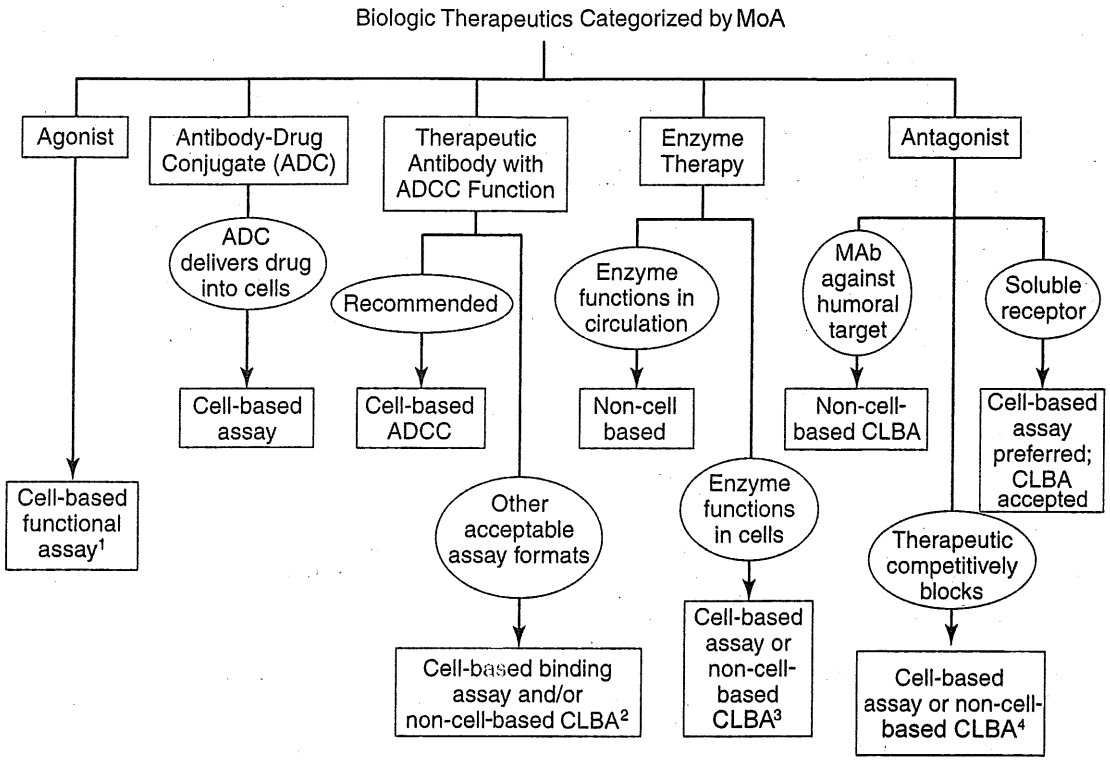

[NOTE—ADCC is "antibody-dependent cell-mediated cytotoxicity;" CLBA is "competitive ligand binding assay;" MAb is "monoclonal antibody."]

[1] A cell-based functional NAb assay is recommended for therapeutic agonists targeting cellular receptor. This includes therapeutics with a nonredundant or redundant endogenous counterpart.

[2] For therapeutics with ADCC function, a cell-based ADCC assay potentially has technical challenges. In addition, ADAs specific to the Fc region of a humanized antibody therapeutic often bind to other nonspecific IgGs in serum matrix and are difficult to detect in a binding ADA assay format. Under this situation, a ligand binding assay using a bridging format or an assay method based on antigen–drug interaction could be a viable platform for the NAb assay.

[3] Non-cell-based assays should be considered, in consultation with a regulatory agency, if the NAb-induced inhibition of lysomal enzyme therapeutics that function at low pH is not feasible under cell-based assay conditions.

[4] For therapeutics that function by binding to more than one receptor, a cell-based assay is recommended.

Figure 1. NAb assay format selection examples.

For agonistic therapeutics that interact directly with cellular receptors, such as growth factors and hormones, development of cell-based NAb assays is appropriate to reflect the MoA of the drug. This category includes therapeutics with a nonredundant endogenous counterpart that may generate clinical sequelae with a high level of risk to patients. For these therapeutics, assays with the capability to determine NAb levels with high specificity and sensitivity are required; the results are generally expressed in titers or equivalents of neutralized drug. These recommendations stem from the concern that NAbs induced by therapeutic proteins can cross-react with and neutralize vital endogenous homologs and cause autoimmune-type deficiency syndromes [e.g., that seen with megakaryocyte growth and development factor (MGDF) and EPO]. Early detection and continued testing for NAbs directed against high-risk molecules are also recommended because NAb data can influence therapeutic decisions.

For antagonistic therapeutics (e.g., anti-IgE or anti-coagulation factors), some regulatory agencies have accepted non-cell-based competitive ligand binding assays for detection of NAbs that are directed against therapeutics with humoral targets. However, for antagonistic therapeutics that interact with a receptor and a coreceptor to exert a pharmaceutical effect on target cells, cell-based assays are generally recommended as this type of MoA cannot be reflected accurately in a non-cell-based ligand binding assay because an ADA that does not block drug–target interactions may interfere with target coreceptor interactions, causing functional neutralization.

In terms of other therapeutics, such as antibody-drug conjugate (ADC), antibody therapeutics with effector functions for clinical efficacy, and enzyme therapeutics that function in target cells, cell-based assays are generally recommended to reflect the drug's MoA. However, for lysosomal enzyme therapeutics that function at pH 4–5, it may be challenging to generate any NAb controls that can inhibit the enzyme at low pH with sufficient sensitivity. In this situation, the development of a

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non-cell-based assay (e.g., inhibition of binding between an enzyme drug and its cognate receptor) is warranted in consultation with a regulatory agency. In addition, a non-cell-based enzyme activity assay could be appropriate for enzyme therapeutics, even those that exert their activities within cells. However, results of such an assay will be conclusive only if the conditions required for the intracellular activity of an enzyme drug also allow for the effective binding of a putative NAb to the drug.

Generally, more frequent NAb testing (e.g., monthly or bimonthly) is recommended for high-risk situations. Sampling should be extended post-study for patients who test positive until they test negative in two consecutive tests at least 2 months apart, or based on the half-life of the ADA (e.g., 3–5 half-lives). This may include post-marketing testing. For medium- and lower-risk situations, testing should be conducted on case-by-case basis in consultation with regulatory agencies.

To fully assess the potential risk associated with NAbs, it is also important to understand the exposure–response relationship of a given biotherapeutic, because this relationship could suggest the extent to which NAbs can affect drug activity in vivo. For example, a high dose of drug would be more difficult to neutralize because more NAb would be necessary; thus, there is a lower risk of efficacy loss. In contrast, when a low concentration of drug is sufficient to achieve therapeutic effect, low levels of NAbs could neutralize drug activity, hence increasing the risk level. Similar situations can occur when the concentration of an endogenous drug counterpart is low; even low levels of NAb could neutralize its physiological function and thereby increase the antibody-related risk.

DESIGN OF NAB TEST METHODS

General Considerations

The selection of a NAb assay type is heavily driven by the MoA of the therapeutic, as well as the nature of its target. An example is a monoclonal antibody therapeutic that binds to a receptor on the cell surface, which in turn affects interactions with other cell surface receptors. For this situation, the analyst should use a cell-based NAb assay because it is unlikely that such interactions could be mimicked on a microtiter plate surface. *Figure 1* shows various selections of NAb assays, categorized by their dependence on the MoA of the therapeutic. NAb assay results are generally reported as quasi-quantitative values using a titer, or are qualitative, with just a positive or negative result. Certain technology platforms report quantitative values, such as a surface plasmon resonance (SPR) assay (see ⟨1106⟩ and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩) or in special cases, neutralization of specific amounts of drug. It could be argued that any numerical value of sample neutralizing capacity is quasi-quantitative when using an antibody preparation as a reference standard, because the affinities and nature of the antibodies within the standard are unlikely to mimic exactly what is contained in a test sample. In addition, comparing sample values between different laboratories that use different standard preparations will likely yield different results for the same sample. Therefore, although a NAb sample value in mass units is helpful for making relative comparisons, all the caveats associated with that value should be considered. A titer value is recommended as the primary readout for NAb assays and is created by testing a sample using serial dilutions. The reciprocal of the test sample dilution that produces a positive result, as defined by the assay cut-point, represents the assay titer. This assay titer should be multiplied by the initial minimum required dilution (MRD; see also ⟨1106⟩), if not accounted for, to report the titer of NAbs present in the neat sample matrix.

Cell-Based Methods for NAb Assessment

As stated previously, cell-based NAb assays can have two general types of readouts: one based on binding events and the other requiring a series of intracellular events that lead to a functional readout. Drug binding to a receptor or surface ligand on the cell can result in its internalization, phosphorylation, or changes in cyclic adenosine monophosphate (cAMP). Functional assays have more complex cellular readouts, such as proliferation, reporter gene expression, or protein synthesis and secretion.

As shown in *Figure 1*, if a cell-based NAb assay is appropriate, it is important to study the performance parameters of the critical components in the assay system, namely the cell line, drug product, positive control (PC) NAb, and test species matrix. Cell-based NAb assays can be technically challenging because of the need to optimize the cell line, culture conditions, and sample matrix components. Lack of optimization can compromise assay precision, robustness, and sensitivity. Initially, the cells used for the drug's biological potency assay could be evaluated for use in the NAb assay. Use of the same cell line provides several advantages in that cell line maintenance and culture conditions have been optimized. Often, this system can be adapted for a biological matrix. Alternatively, commercial sources of cell lines are available, or a drug-responsive cell line could be genetically engineered. Cell lines transfected with target receptors can offer the advantage of more controlled receptor density, specificity, and enhanced signaling. Reporter gene expression cell lines can provide sensitive assay endpoints.

The cells' responsiveness to the drug should be characterized, particularly before and after freezing, as well as during continuous culture (see also general information chapter *Cryopreservation of Cells* ⟨1044⟩). Drug dose-response experiments in the appropriate biological matrix (e.g., serum) can identify early interference issues. Ideally, several matrix samples should be evaluated. Specificity should be studied to allow use with serum from the species of interest that developed NAb after exposure to the drug. PC antibodies are often isolated from antisera of this species, but because this is not always available during assay development, a surrogate control antibody reagent is produced to use during assay development and validation (see the section *Development of Positive and Negative Controls in NAb Assays*). Cell-based assay formats that have been used successfully in drug product development are shown in *Table 1*. The type of assay format chosen is influenced by the mechanism by which the drug interacts with cells. These interactions can either be direct or indirect. A direct interaction is one in which the drug product exerts its effect by acting directly on cells, such as with cytokines, peptides, or monoclonal antibodies to cell surface determinants. Indirect interactions are those where the drug product blocks the interaction of a ligand with its cell surface receptor and consequently interferes with the biologic activity of the ligand. Examples of such drugs include therapeutic monoclonal antibodies to soluble factors and soluble receptors.

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Table 1. Frequently Used Cell-Based NAb Assay Formats

Assay Endpoint	Assay Platform Examples	NAb Action	Advantages	Disadvantages
Cell surface interactions	Fluorescence cytometry, plate-based cell surface binding	Interferes with binding of drug to target site on the cell, or affects internalization of drug product into the cell.	Drug, target, and NAb interaction occurs only on the surface of cells, which may result in simple and robust assays.	Does not apply to drugs with a MoA involving signaling pathways within cells; inadequate receptor expression on cell may be a limitation.
Phosphorylation of intracellular substrates	Kinase receptor activation, enzyme-linked immunosorbent assays (ELISAs), intracellular staining	Affects drug-induced phosphorylation of target receptor/substrate, or affects ligand-induced phosphorylation by blocking drug.	Utilizes early event in cell signaling, which may result in a shorter assay.	Needs phosphorylation site-specific antibodies; multiple assay steps are needed if using ELISA to detect phosphorylation.
Cell proliferation	^{3}H incorporation, Alamar Blue	Affects cell proliferation that is induced or inhibited by drug, or affects ligand-induced cell proliferation by blocking drug.	Well-established assay endpoint for growth factor-type drugs reflects what happens to the cells.	Assay endpoint is the outcome of multiple intracellular pathways and steps, which may result in a long assay; prone to interference by serum factors; assay needs to show specificity.
Reporter gene expression	Luciferase, β-galactosidase, chloramphenicol transacetylase	Affects reporter gene expression by drug product, or affects ligand-induced reporter gene expression by blocking drug.	Assay can be quick, sensitive, and robust.	Need to invest time to construct reporter gene cell line that can respond robustly in presence of test species serum; need to demonstrate specificity.
Protein expression or secretion	ELISA, enzyme immunoassay, radioimmunoassay, electrochemiluminescent assay (ECL), scintillation proximity assays	Influences protein expression by drug, or influences ligand-induced protein expression by blocking drug.	The protein can be measured in the cell supernatant or in cell lysate by using an ELISA.	Assay endpoint is the outcome of multiple intracellular pathways and steps, which may result in a long assay; assay involves cell culture followed by ligand binding assay to detect synthesized protein, thus two assays are required.
Effector functions	Complement-dependent cytotoxicity (CDC), ADCC, Fcγ receptor binding	Influences target cell fate mediated by effector functions of therapeutic antibodies.	Required for antibody therapeutics where both Fab and Fc domains are involved in function.	Multiple effector functions (ADCC, CDC, phagocytosis, etc.) may be involved, which can make assay choice, assay development, and interpretation of results challenging.
Cytotoxicity/apoptosis	Luminescent cell viability assays, terminal deoxynucleotidyl transferase dUTP nick end labeling assay	Affects cell cytotoxicity/apoptosis induced by drug, or affects ligand-induced cell cytotoxicity/apoptosis by blocking drug.	Directly reflects MoA of cell killing mediated by therapeutics.	Needs to demonstrate specificity.

Consideration is given to the assay endpoint, which can reflect either early or late cellular responses. Early responses include the initial binding to the cell surface, internalization, an early event in cell signaling, or gene expression. Late responses reflect the outcome of multiple intracellular pathways that result in a biological outcome such as proliferation, induction of measurable products, apoptosis, or effector functions such as ADCC or CDC.

Care should be taken to optimize the concentrations of critical assay components that influence its sensitivity for detecting NAbs. In the case of a direct NAb assay, the dose response at several concentrations of the drug product should be evaluated. The amount of drug product used in the assay should be within the linear portion of the dose-response curve that produces a reproducible response (e.g., often a 30%–80% maximal assay response). The sensitivity of an indirect NAb assay depends on both the ligand concentration and the drug product concentration.

In all cases, the appropriate controls should be incorporated. For example, in a direct assay format in which the drug acts directly on the cells to induce a biological response, the assay controls should include cells alone, cells with drug product, and cells with drug product and PC NAb. An indirect assay format should include cells alone, cells with ligand, cells with ligand and drug product, and cells with ligand, drug product, and PC NAb. The assay can be set up as a single dilution to provide qualitative information (positive or negative), as is generally the case for preclinical studies, or can use multiple dilutions to give a quasi-quantitative readout, such as the titer. Designing well-controlled assays facilitates monitoring of assay performance over time and identification of factors to consider as root causes of assay failure.

Non-Cell-Based Methods for NAb Assessment

Cell-based functional assay formats have traditionally been recommended by the regulatory agencies for NAb assessment. However, in comparison to the technical challenges of cell-based assays described above, non-cell-based immunoassays are capable of overcoming some of the technical limitations inherent to the cell-based bioassays, and have therefore become another useful technology platform for NAb evaluation. Non-cell-based immunoassay platforms, especially competitive ligand binding assays (CLBAs) when relevant to the MoA of the drug, can be used for NAb assessment if proven to specifically detect NAbs (*Figure 2A* and *Figure 2B*).

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For a range of biological therapeutics (e.g., antagonistic MAb therapeutics against a soluble ligand), the drug exerts its pharmaceutical effect by binding to its target and blocking the interaction of the ligand with its cell surface receptor; consequently, the drug interferes with the biological activity of the ligand. In this instance, a non-cell-based assay method is appropriate for NAb assessment because the assay method reflects the MoA of the drug by measuring drug binding to its target and inhibition of such binding activity by NAbs.

The CLBA-based NAb assay design is based on the competition between the ligand and NAbs for a limited number of binding sites on the ligand binding therapeutic. As the level of NAb increases, less ligand binds to the drug and the measured assay response decreases when compared to a control sample that does not contain any neutralizing activity. Therefore, within the linear range of the assay, the NAb concentration is proportional to the percentage change in assay response. In theory, any ligand binding assays, such as solid- or liquid-phase immunoassays (e.g., radioligand, ELISAs, chemiluminescence, and ECL assays), radioimmunoprecipitation assays (RIPA), and SPR, may be adapted to the CLBA format for the detection of NAb in test samples (see *Appendix* and *Immunological Test Methods—General Considerations* ⟨1102⟩ and *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and ⟨1105⟩ for more information).

Two assay formats are available for non-cell-based CLBA (*Figure 2A* for direct format and *Figure 2B* for indirect format).

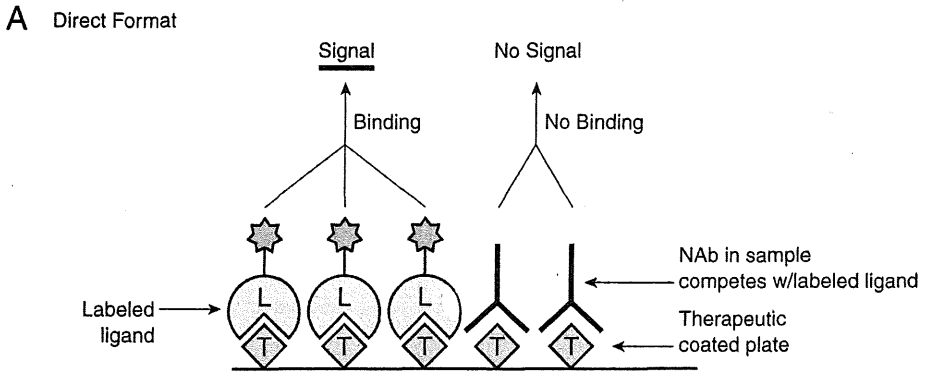

Figure 2A. Direct CLBA format: In this assay format example, the therapeutic product is coated on a plate and serves as a capture molecule to bind to the ligand labeled with a detection molecule (e.g., an enzyme, a fluorescent label, or an ECL label). Binding between the therapeutic and the ligand is inhibited when NAb is present in test samples, resulting in a lower signal. "T" represents "therapeutic product" and "L" represents "ligand."

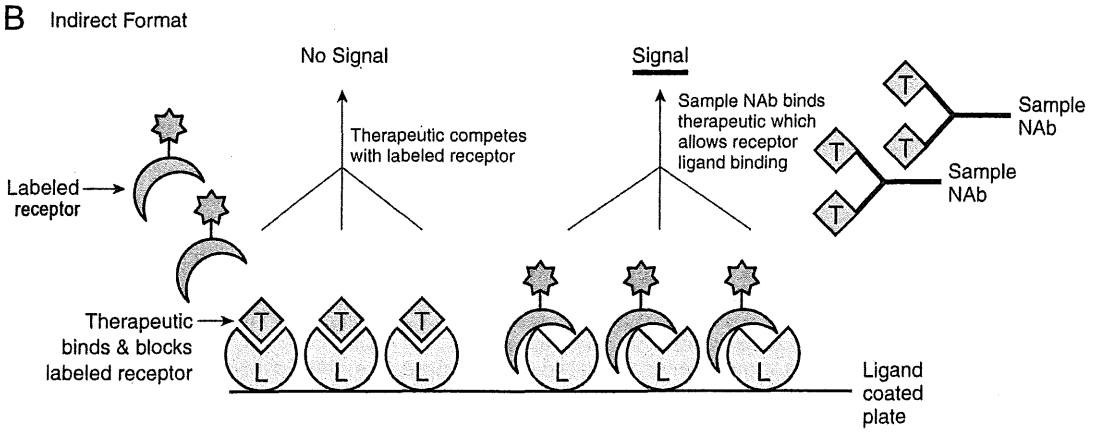

Figure 2B. Indirect CLBA format: In this assay format example, the ligand is coated on the plate, and the therapeutic competes with the labeled receptor for binding to the ligand. When NAb is present in test samples, it binds to the therapeutic and the neutralized therapeutic is unable to bind to the ligand; therefore, the signal will increase because the labeled receptor is now able to access and bind to the ligand.

The direct CLBA NAb assay format, based on measuring the binding of a drug to its target, is a simple approach (*Figure 2A* and *Table 2*), whereas the indirect CLBA NAb assay format monitors drug-mediated inhibition of ligand–receptor binding (*Figure 2B* and *Table 2*). The two assay formats both utilize the MoA of the drug (inhibition of drug–ligand or ligand–receptor binding) but measure the neutralizing activity using different assay endpoints.

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Table 2. CLBA NAb Assay Format and Critical Assay Components

Assay Format	Assay Measurement	Assay Design for Solid-Phase Immunoassays	Assay Components	Advantage	Disadvantage
Direct CLBA	Binding of a drug to its ligand (target)	The drug as capture molecule and the labeled ligand as detection molecule (the reverse format is more prone to drug interference)	Unlabeled or labeled drug, conjugated target, test species samples, positive and negative controls	Simple assay design	If the binding prevents signaling or attachment of another ligand to its receptor, only the very early binding step is reflected in this assay.
Indirect CLBA	Drug-mediated inhibition of ligand–receptor binding	The ligand as capture molecule and the labeled receptor as detection molecule (the reverse format is feasible)	Unlabeled or conjugated ligand, conjugated receptor, unconjugated drug, test species samples, positive and negative controls	Reflects a consequence of NAb activity that is further downstream.	The complexity of receptor purification makes it challenging to maintain proper protein folding and consistent assay performance.

For the direct CLBA NAb assay format applied in a solid-phase immunoassay, the immobilized drug usually serves as the capture molecule, while the labeled target serves as the detection molecule, generating an assay signal after it binds to the drug. The neutralizing activity can be assessed as the level of inhibition of drug-target binding when NAbs are present in test samples. The assay sensitivity of the direct CLBA NAb assay format is largely determined by the drug concentration selected. A lower drug concentration typically leads to a more sensitive NAb assay, but a low dynamic range may limit the detection of a broad range of NAbs. Therefore, it is important to optimize the drug concentration during assay development. To further optimize the assay, implementation of design of experiment (DoE) can be considered to systematically assess the interactions among key assay-operation parameters and identify the most optimal assay conditions.

In certain cases, such as when the ligand or receptor is a highly charged molecule and tends to bind nonspecifically to surfaces, the assay format also can be reversed by using the ligand or receptor protein as the capture molecule and the drug as the detection molecule. However, because of the use of the ligand as a coating agent, this reverse format can be much more prone to drug interference even if comparable assay sensitivity is achieved. Therefore, the direct CLBA NAb assay format using the drug as the capture molecule and the conjugated ligand as the detection molecule is usually preferred.

An indirect CLBA NAb assay format, which is based on drug-mediated inhibition of ligand–receptor binding, also can be used for the detection of NAb to antagonistic therapeutics that neutralize soluble ligands. One applicable format for an indirect CLBA NAb assay uses the ligand as the capture molecule and the conjugated receptor protein as the detection molecule in a solid-phase immunoassay. The reverse format also may be feasible. The immobilized ligand binds to the conjugated receptor, generating an assay signal. Ligand–receptor binding is inhibited in the presence of drug, but occurs when the drug is neutralized by NAb. The neutralizing activity in the test sample is thereby estimated by the level of restoration of ligand–receptor binding when drug function is blocked by NAb.

The sensitivity of the indirect CLBA NAb assay is largely dependent on the concentrations of both the ligand and drug. A lower ligand concentration will result in a lower drug concentration selected for the assay, leading to a higher NAb assay sensitivity. Other operational parameters also can be effectively evaluated through the DoE approach to determine the optimal assay conditions.

When an oligomeric receptor protein is included in the non-cell-based NAb assay, specific consideration should be given to the challenges presented by this type of receptor, which may be less likely to retain the original conformation necessary to bind to the ligand when not associated with the cellular membrane. In addition, cellular receptors typically have one or multiple transmembrane domains that need to be truncated to facilitate receptor purification. Therefore, concerns arise regarding proper protein folding of these truncated ectodomains and retention of the structure necessary for ligand–receptor binding. The complexity of receptor purification further requires that lot-to-lot variation and stability of the protein products be effectively managed in order to maintain consistent assay performance.

Positive and negative controls are critical for monitoring NAb assay performance. Unlike cell-based NAb assays, the design of assay controls for non-cell-based CLBA assays is usually more straightforward, without the need to include background controls. The selection of positive and negative controls is described in detail in the section *Development of Positive and Negative Controls in NAb Assays*.

VALIDATION OF NAB ASSAYS

As described above, there are two main formats for NAb assays: cell-based functional assays and binding-based assays. The various components of validation that should be carried out prior to study initiation are described in the sections that follow. The principles of each aspect of validation apply to both assay formats unless noted otherwise.

Minimum Required Dilution

Determination of the appropriate dilution of assay matrix is an important part of NAb assay optimization because this will affect the minimal test sample dilution and therefore the assay sensitivity. The considerations for defining the minimum-required dilution (MRD) used for the ADA screening immunoassay described in ⟨1106⟩ also may be applied to NAb assays. The effect of sample matrix on assay capability should be evaluated at multiple dilutions, preferably by using different pools of the test species matrix. The dilution of sample matrix that has a minimal effect on the assay response should be selected and further evaluated using the PC antibody spiked into sample matrix. A NAb assay should be able to detect antibodies in the presence of assay

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matrix components that may be expected to be present. These might include complement, coagulation factors, soluble targets, lipids, concomitant medications, and the endogenous homologous counterpart, as well as the administered drug product. As described in ⟨1106⟩, the MRD can be objectively determined and defined as a dilution level that achieves an optimal signal-to-background ratio with acceptable variability.

Factors such as soluble targets that either bind the drug product or act directly on the cells can interfere in the assay, leading to false positive results. Alternatively, certain factors may alter the assay response in a manner that masks the presence of a NAb in the sample (e.g., as noted with the presence of interfering levels of biotherapeutics or growth factors). Confirmation of assay specificity can help to determine the presence of such interfering substances (see the section *Assay Specificity*). Assessment of matrix effects during assay development can be accomplished using pooled matrix. Ideally, the assay matrix should be defined using multiple individual matrix samples because of heterogeneity of the various possible interfering substances. Diseased-state (treatment-naive) matrix may contain additional factors that interfere in the assay and should be evaluated whenever possible.

If lipemic, hemolyzed, incompletely clotted, and, preferably, disease-state sera from naive subjects are available, they should be screened in the assay both with and without the addition of the PC NAb. If the unspiked sera give a response in the assay, and/or the sera interfere in the detection of the control, the analytical procedure should state that samples compromised in such a manner may not yield reliable results and may have to be excluded from testing.

Development of Positive and Negative Controls in NAb Assays

NAb assays, including cell-based functional assays and non-cell-based immunoassays, are designed to detect heterogeneous and often polyclonal anti-drug immunoglobulins. Because of the diverse nature of immune responses to the drug, it is not possible to generate a true NAb reference standard. Assay performance is therefore monitored by utilizing surrogate positive and negative controls (PCs and NCs). Typically, an NC sample is generated by pooling relevant matrix that is negative for ADA, such as matrix collected from subjects with no previous exposure to the drug. One should consider whether the NC pool can appropriately represent the target study population, such as by comparing assay response produced by the NC to the mean response produced by the individual target population matrix samples (at least 20 individuals). In some cases, it can be difficult to obtain a sufficient number of study population-relevant matrix samples or to ensure that samples used in assay validation accurately represent matrix characteristics of the study population samples. The incurred study baseline samples should be assessed for any evidence of predose reactivity to the compound.

NAb assay PCs typically consist of a hyperimmunized animal serum, a monoclonal Ab, or material that otherwise has specific, and generally high, affinity neutralizing ADA reactivity. The antibody preparation used to generate a NAb-assay PC must be able to neutralize the biological activity of the drug compound in vitro. Ideally, the PC may be prepared by spiking immunoaffinity-purified ADA (polyclonal or monoclonal), or a protein-A/G purified preparation spiked into an appropriate neat matrix may be used. Alternatively, for preclinical studies only, whole hyperimmunized animal serum spiked into appropriate neat matrix can be used. For MAb biotherapeutics, the PC generally has anti-idiotypic antibody reactivity. The PC antibody is considered a critical reagent and should be documented and characterized for use in the assay. Pertinent documentation and evaluations that should be considered include immunization scheme, purification procedure and yield, protein concentration, relative affinity, cross-reactivity, isotype, and NAb titer. Thorough characterization will promote reagent consistency over time and minimize lot-to-lot variation. Refer to ⟨1106⟩ for stability monitoring of assay controls.

Although PCs are used to develop, validate, and monitor performance of NAb assays, these controls are not assumed to represent actual incurred study samples. A great diversity of immune responses to drug molecule, with a broad range of binding affinity and specificity characteristics, should be expected. Therefore, PC and NC performance is used primarily to ensure that the assay performs as expected (i.e., to confirm system suitability).

Assay Cut-Points

The cut-point of a NAb assay is the assay response used to determine whether a sample is positive for neutralizing activity. All individual drug-naive subject samples and NC samples used for the cut-point evaluation should be spiked with a fixed concentration of drug determined prior to validation; however, this would not apply to the direct format for non-cell-based NAb assays. The assay response can be expressed as assay signal or as the ratio of assay signal from a test sample to that derived from the NC. The NAb-positive samples would have a response above the cut-point if NAb increases the assay response and below the cut-point if NAb reduces the assay response. Alternatively, the assay response may be normalized and computed as percentage change from the NC.

Because ⟨1106⟩ includes an outline of the cut-point evaluation process, this chapter only provides a summary of the evaluation process, with emphasis on and clarification of some key steps for NAb assays (additional helpful resources can be found in the *Appendix*). Alternative statistical methods can be applied in some steps of the cut-point evaluation process (e.g., outlier evaluation) with appropriate justification.

Based on statistical and practical considerations, at least 30 individual subject sera from the target disease population (if available) or healthy donors should be used for cut-point evaluation. Samples from these subjects should be tested over at least three independent runs (e.g., days, analysts) by at least two analysts using a balanced-design framework. At least three reportable results for the negative control should be available from each plate, where a reportable result may be the average of duplicate sample results if the study samples are also tested in duplicate. Also, these three results should come from different locations in the plate, such as the first column, middle column, and last column of the plate.

If the cut-point is estimated from only healthy donor sera during this validation phase, data from the target disease population should be evaluated during the in-study phase to determine statistically whether the distributions of assay response are similar to those of the healthy population. If the variances are significantly different, the cut-point should be re-evaluated using the target disease population. If only the means are significantly different, the same cut-point can be used after redefining the NC based on the target population. Other population differences relevant to the clinical study with respect to gender, age, and other factors also should be considered in the sample selection process.

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The distributions of original data and log-transformed data (or other transformations), averaged across assay runs, can be evaluated in terms of the skewness coefficient and normal probability plot. Data from the scale (e.g., original, log) that provides the most symmetric or close-to-normal distribution should be used in all subsequent analyses, such as outlier evaluation, cut-point calculations, and comparisons of means and variances across assay runs. Analytical and biological outliers should be identified using appropriate statistical methods such as using the conditional residuals and subject mean estimates from a mixed-effects model that includes the relevant sources of variation in the cut-point experiment (e.g., this model may include Subjects nested within Subject Groups, Run Number nested within Analyst, and Plate ID as random-effects, as well as Subject Groups, Analyst, Plate Testing Order, and the interaction of Analyst and Plate Testing Order as fixed-effects).

The analytical outliers should be identified and removed before identifying the biological outliers, and the distribution of assay response should be evaluated after removing the analytical and biological outliers. If the distribution is adequately normal (i.e., Shapiro–Wilk test is not significant), the parametric approach [mean +2.33 × standard deviation (SD), if NAb increases the assay response, and mean −2.33 × SD, if NAb reduces the assay response] can be used for determining the cut-point from these validation data. This calculation will yield a 1% untreated false positive rate from a normal distribution. The cut-point may be defined in terms of other false positive rates, such as 0.1%–5% on a case-by-case basis, with appropriate justification and discussions with regulatory agencies. If the distribution is not adequately normal (i.e., Shapiro–Wilk test is significant) but is still symmetric enough (skewness coefficient <1), and the departure from normality is mostly due to the heavy tails or some extreme values that may not have been identified as statistical outliers, robust alternatives to the mean and SD, such as the median and 1.4826 times the median absolute deviation, respectively, can be used in the above cut-point calculation formula. If the distribution is highly nonsymmetric (skewness coefficient >1), the nonparametric 99th percentile is recommended, although this should be a last resort as it requires a much larger number of subjects (>100) to obtain a reliable estimate.

Another important consideration is that the SD estimate used in the cut-point estimation should include all the different sources of variation that are relevant to the context where study samples will be tested during the in-study phase. Because study samples are typically tested by multiple analysts over several plates and assay runs, the SD used in the cut-point evaluation in such cases should include, at the minimum, interanalyst, interplate/run, intraplate/run, and intersubject variability.

In order to understand the nature of variability in the assay, and to determine whether the same cut-point evaluated during the validation phase can be used for identifying samples with neutralizing activity during the in-study phase, the means and variances of the distribution of the assay signal from approximately six runs should be compared using a mixed effects model and Levene's test, respectively (see ⟨1106⟩). If these are not significantly different, then the same cut-point (fixed cut-point) can be used during the in-study phase. Otherwise, the cut-point evaluated as above using these validation data should be divided by (or subtracted from) the NC. This is called a multiplicative (or additive) normalization factor. This factor may be multiplied (or added) to the NC used during each run of the bioanalysis phase to define the run- or plate-specific cut-point. Such a cut-point is called the floating cut-point. If the original data are found to be approximately symmetric or normal, then an additive correction factor may be used. If a log transformation is necessary to ensure approximate symmetry or normality of the distribution or if the distribution of the ratio of original sample results (assay signal) to NC has been shown to be adequately symmetric or normal, then a multiplicative correction factor may be used. In such cases, all of the analyses for cut-point evaluation also may be done in terms of the ratio of assay signal from the individual subject sample to the NC from the corresponding plate.

In practice, regardless of whether the means and variances are significantly different between assay plates or runs, the use of a floating cut-point is recommended as it is more accommodating to minor drifts between assay runs during the in-study sample testing phase.

If a fixed cut-point is justified from the above evaluations and therefore is implemented during the in-study phase, a higher level of attention is required to monitor and validate changes in reagents and other assay conditions. Because the cut-point has been fixed in relation to that assay as it existed at the time of the experiments that determined the cut-point, it is essential to ensure that the assay signal results from the controls and test samples are consistent and stable in the event that any changes are made to the assay (e.g., new reagents, analysts, or machinery).

System Suitability Criteria

NAb assays typically have an intricate design with multistep operations. These assays use complex reagents and equipment and also require extensive data collection. It is therefore important to conduct system suitability assessments in order to evaluate and verify overall method validity and utility.

Specifically, negative and positive assay controls and additional background controls (e.g., cells alone and/or cells with drug product) should be included as part of each analytical run during assay validation and during the sample testing phase. The exact nature of background controls will depend on the type of NAb assay developed (see the section *Design of NAb Test Methods*). Data obtained during assay validation are used to develop assay acceptance criteria.

Generally, monitoring performance of the PC at low level (LPC) and high level (HPC) is most critical. Inclusion of the LPC helps monitor the established assay sensitivity. Exclusive use of a mid-level PC (MPC) should be avoided because the middle range of the assay response versus PC concentration may not be affected as significantly by changes in assay conditions (e.g., reagent, changes) as are LPC and HPC. Alternatively, performing an analysis of a PC tested in a dilution series provides coverage of multiple PC levels, including LPC. In some circumstances, use of an NC that includes a non-neutralizing antibody may be helpful for identifying run failure and preventing reports of false positive results. Typical acceptance criteria for the assay control samples include (a) precision of the raw assay signal for PC and NC samples, (b) performance of the LPC sample or reportable titer value for the PC, and (c) upper and/or low limit for the assay response generated by the assay NC. Other criteria may be applied.

When the floating cut-point approach is used for the NAb assay cut-point evaluation, the system suitability criteria or limits can be defined for the ratio of the LPC to the NC and for the ratio of the HPC to the LPC, instead of defining limits separately for each PC. For the in-study phase, it is also useful to apply acceptance criteria for intra-assay precision (variability of response of replicates in an assay). As discussed in ⟨1106⟩, setting criteria for passing or failing assays in pre-study validation experiments should be avoided and all assays performed during pre-study validation should be included except for those rejected for an

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assignable cause. The appropriate choice of LPC concentration and the acceptable range of performance are important for ensuring the ability to monitor sustained assay sensitivity. The failure rate for LPC is expected to be 1% based on the PC performance. Similarly, an assay failure rate can be set at 1% based on the performance of the NC. If applicable, higher rates of failure (e.g., 5%) may be used. To set a specific failure rate based on the NC and LPC sample performance, appropriate assay response limits should be calculated based on control performance during assay pre-study validation. Alternatively, limits may be established for the PC titer value. These should be defined based on the PC performance observed during the pre-study assay validation phase. Importantly, depending on the specifics of the NAb assay format, the signal in the assay is expected to increase (*Figure 3A*) or decrease (*Figure 3B*) in response to the increasing PC concentration. Hence, an appropriate NC limit should be set. For example, in the first case, where the assay signal is increased, an upper limit for the NC should be established. In the second case, a lower limit for the NC will be most important.

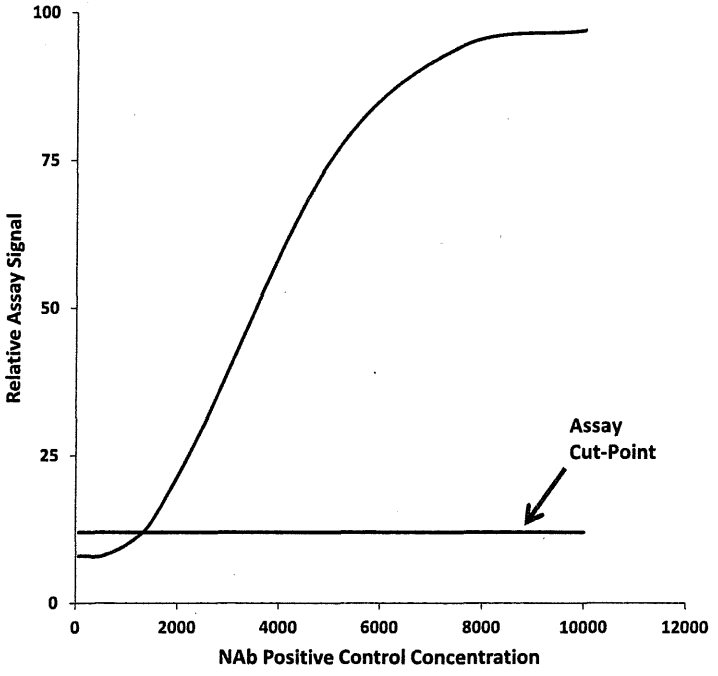

Figure 3A. NAb assay response as a function of PC concentration. Assay signal increases with increasing PC concentrations.

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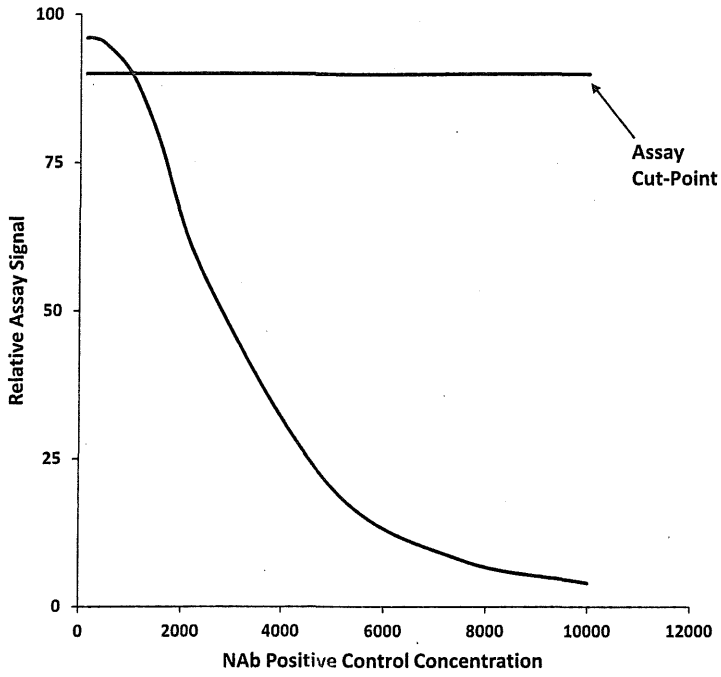

Figure 3B. NAb assay response as a function of PC concentration. Assay signal decreases with increasing PC concentrations.

Initial assay control acceptance criteria may be based on the existing information about assay performance obtained during the assay development and qualification phase. Alternatively, standard criteria that are based on the specific, existing NAb assay validation standard operating procedure may apply. Important technical details such as instrument signal readout capabilities should be considered when setting expectations for the assay control performance. Actual criteria for the assay controls can only be determined after a holistic evaluation of the assay validation information in its entirety.

Because of the complexity of the NAb assays, specifically the cell-based NAb protocols, an effort should be made to identify particular steps and conditions with the highest potential to affect assay performance. Generally, assay control performance should be monitored during the study support phase to ensure consistency of reported data. Assay control performance data should be monitored for any short- or long-term trends and variations relative to the predefined range. If a suspicious trend in the control sample performance is identified or the signal falls close to a limit of the acceptable range, an investigation should be considered.

Relative Sensitivity

NAb assay sensitivity is defined as the lowest concentration of an assay PC antibody that can be detected reliably by the method. The result obtained for this validation parameter is highly dependent on the characteristics of the PC used to conduct the experiments, including its neutralizing capacity and binding affinity to the drug. The relative sensitivity of NAb assays is also inversely dependent on the concentration of drug used in the assay.

To conduct the experiment, PC is spiked into assay-relevant pooled matrix. These spiked samples are tested in multiple runs, commonly in at least three independent runs by two operators for a total of six runs. It is recommended that more than one antibody curve be performed per run per operator.

Generally, linear interpolation between values just above and below the assay cut-point is conducted to calculate the assay sensitivity parameter. In some cases, a four-parameter model is used for which at least six values generated by various concentrations of PC should be available to appropriately analyze the resulting data set. One of the values should fall below the assay cut-point. When using linear interpolation or a four-parameter-fit approach, the PC concentration that would generate an assay response equal to the cut-point value is calculated. Values generated in multiple tests over multiple days are averaged, and the result is reported as the relative sensitivity of the assay. Once established, the assay sensitivity value can be used to guide selection of a LPC concentration to be used when monitoring assay performance during assay validation and when testing incurred study samples. Generally, the LPC concentration is chosen so that the rate of assay failure due to performance of the PC is NMT 5%.

Because the assay sensitivity depends greatly on the characteristics of the assay PC, assay sensitivity will vary between different NAbs. The assay sensitivity value determined during assay validation cannot be used to predict the actual NAb concentration that could be detected in study samples. The NAb assay sensitivity parameter is useful when evaluating various analytical platforms, during assay development and optimization, and for selecting appropriate PC concentrations for assay validation and system suitability testing. Typical targets for the NAb assay sensitivity are often 0.5–2 µg/mL; however, because NAb assays are complex, a case-by-case, fit-for-purpose approach to selecting an appropriate assay sensitivity is used with the goal of detecting clinically relevant NAbs.

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Assay Specificity

Assay specificity is defined as the ability to unambiguously detect the analyte of interest. For example, in the case of cell-based assays, an initial assessment should be done to investigate the ability of the chosen cell line to respond to biological components expected to be present in the assay matrix that may structurally or functionally resemble the drug molecule or its molecular target; the goal is to ensure the specificity of the NAb format. Such an assessment may aid in determining the assay format, the analytical platform, the type of cell line to be used in the assay, any sample pretreatment, and other assay conditions. Careful attention should be paid to potential differences between normal and disease-state samples. Assessment of assay specificity may include the testing of irrelevant ADA (e.g., antibodies to other similar molecules) and should include, where possible, specific binding ADA that are known to lack neutralizing activity. The comparison may be made either by evaluating an antibody dilution profile or by spiking the antibody in excess into a pooled negative matrix sample. The irrelevant antibodies are not expected to score positive in a well-designed, specific NAb assay. Other matrix components (e.g., soluble forms of receptors or other drug binding partners) may exhibit inhibitory effects on the drug activity. It is important to understand whether true confirmatory testing should be included as part of an incurred sample analysis routine. Such confirmatory testing should demonstrate that the inhibition of the drug activity is specific to NAbs and not other factors found in the assay matrix. NAb confirmatory testing, also referred to as matrix interference assays, generally employ a cut-point-based approach. If a decision is made to include a matrix interference NAb confirmatory assay during routine sample testing, the validation should follow the general principles described above for the assay cut-point determination.

Selectivity and Interference

Selectivity evaluates the ability of a NAb assay to detect a NAb PC in a matrix sample containing potential interfering factors. These matrix factors may bind to NAb through specific or nonspecific interactions, interfering with NAb detection. General matrix interference can be investigated by evaluating the recovery of the NAb assay response generated by the HPC and LPC prepared in 10–20 individual relevant matrix samples.

One of the major interfering agents in a NAb assay is the drug itself, when present in test samples from dosed subjects. The drug interferes with the ability of the assay to detect NAbs, causing false negative or false positive results, depending on assay design. The magnitude of drug interference is dependent on multiple factors such as the circulating drug concentration, the concentration and other characteristics (affinity, avidity) of the PC antibody, the half-life of the drug, and the assay design. Therefore, it is not possible to establish a universal "drug tolerance level" for all NAb assays. Nevertheless, drug interference in the NAb assay should be addressed in the assay design and testing strategies. During assay development or optimization, drug interference may be assessed by adding titrated concentrations of drug into undiluted matrix containing fixed concentrations of a positive NAb control, based on assay sensitivity. The limit of drug tolerance is reported as the highest concentration of drug at which PC NAb remains detectable, where detectability should be defined (e.g., a certain signal-to-noise ratio, a selected level above background). To ensure that the assay method is sensitive enough to detect NAbs in the presence of circulating drug, the positive NAb control could be titrated in undiluted pooled matrix sample (e.g., at 250 ng/mL or 500 ng/mL for clinical studies or 1000 ng/mL for nonclinical studies) to assess drug tolerance level for the assay method. Thus, based on the sensitivity of the NAb assay, the PC should be diluted to that level when trying to detect NAb in the presence of circulating drug. The drug tolerance level can vary considerably when different concentrations of the positive NAb control are added to the assay matrix. NAbs may also differ in affinity and/or avidity. Therefore, the drug tolerance level assessed using a PC may not predict the actual levels of drug interference in study samples.

Because NAb assays tend to be highly susceptible to drug interference, it is generally not recommended to test for NAbs in samples from time points when drug levels are expected to be high. However, it may be necessary to analyze NAb activity in study samples containing circulating drug to investigate when the onset of the NAb response occurs and its impact on drug exposure. Under these circumstances, methods need to be applied to overcome drug interference and enable NAb detection in the presence of high levels of circulating drug. Strategies commonly used to improve drug tolerance level include acid dissociation and removal of excess drug through physical separation or solid-phase absorption. An alternate approach is a drug quantitation-based approach in which samples are tested for the bioactivity of circulating or exogenously added drug. Each method has its own caveat. For example, acid pretreatment may affect the activity of NAbs. In cell-based NAb assays, excess acid contained in samples may negatively affect the cellular response and decrease the assay signal, thereby compromising the advantage offered by using the acid dissociation procedure.

In addition to drug interference, soluble drug ligands may also interfere with NAb assays, potentially generating false positive results. Target interference also can be introduced when the drug target is released from drug–target complexes during acid dissociation. These interfering factors may be addressed by optimizing sample pretreatment methods. One feasible approach is to pretreat the samples with beads conjugated to an anti-target antibody. After removal of beads, the target-depleted samples can be used in the NAb assay.

Precision

Precision—intra-assay and interassay—is the quantitative expression of variability, and it provides a measure of the amount of random error that occurs during the execution of an analytical procedure. Precision estimates are useful indicators of assay performance in the specified assay matrix.

QUALITATIVE NAb ASSAYS

Per general information chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1), intra-assay precision (repeatability) is the degree of agreement between results generated by consecutive analysis (replicate testing) of the same assay controls or samples under the same operating conditions, by the same operator using the same equipment in a laboratory,

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within a short period of time. Four to six independent preparations of LPC, HPC, and/or NC samples in a single lot of pooled donor serum (normal or disease state) are evaluated in duplicate or triplicate, in multiple positions on the same plate in a randomized manner, to determine the relevant sources contributing to response variability. The imprecision of the assay response (optical density, fluorescence unit, luminescence unit, or percentage change in assay signal after normalization or interpolation), is calculated and reported as percent coefficient of variation (%CV), which equals (SD/mean) × 100. The %CV values of the mean assay response obtained with the various assay controls should suffice for assessment of intra-assay precision. The %CV may vary depending on the technology used for detection, assay methodology, and procedural complexity. The expected target CV or pooled %CV for intra-assay precision should therefore be defined based on assay capability, as well as on intended use. It is not possible to generalize acceptable precision as it depends on the use of the assay and drug type, risk to patient, and other factors, but the need to rely on the assay result should drive acceptable limits.

Interassay precision (also called intermediate or total precision) encompasses within-laboratory variation among assay runs, and therefore represents the overall precision of the assay. The experiment described above should be executed over multiple days with at least two operators, especially if the sample testing will be executed by more than one operator in the study phase. The pooled intraplate SD and the SD of the mean for each sample tested on multiple plates over multiple days can be used to calculate the intermediate precision, assuming that most of the variability is attributable to plate variability and that the sample size is the same on every plate. Intermediate precision is highly dependent on the assay methodology and procedural complexity. The target intermediate precision, therefore, should be defined based on assay capability, as well as on intended use (fit-for-purpose).

Using an alternate approach, interassay precision may be assessed by deriving the mean, SD, and %CV of the NCs and PCs from all of the experiments conducted during assay development (provided that the plate location effects are negligible), with a few exclusions. The runs to be excluded are those with an assignable operator or equipment error, or with method variations introduced intentionally for robustness testing.

QUASI-QUANTITATIVE NAb ASSAYS

To assess the precision of the reported titers, operators generally use the LPC and one to two concentrations of the HPC. The HPCs (minimum of three independent preparations) should be diluted in a two- or three-fold titration series using undiluted pooled assay matrix as the diluent and then should be tested in the assay. The HPC can also be diluted in MRD matrix pool, as long as future samples are diluted in the same manner. To measure intra-assay precision, it is generally recommended that three titration curves of the low and high PC be analyzed by one operator on the same day. To measure interassay precision, three titration curves each of the low and high PC should be analyzed on a minimum of 2–3 different days by two operators. Titers are determined as a reciprocal value of the highest dilution of the PC that tests positive. Target titers can be determined and assigned to each low and high PC or can be calculated as mean values by averaging the titer values obtained for the low and high PCs in the precision assessment. Intra-assay and interassay precisions of titers are then evaluated by comparing titers obtained for individually prepared curves to the target titer assigned for the low PC and high PC, respectively. As described in ⟨1106⟩, a recommended but more rigorous approach is to use these data to define a minimum significant ratio (MSR). The calculated MSR reflects the smallest fold-change in the titer values that can be considered statistically significant ($P < 0.05$); for instance, if MSR = 5, then titers that are different by more than five-fold can be considered significant.

In general, the acceptance criterion for titer precision is that the assigned titer value should be within one dilution of the target titer in independent titration series. This, however, will depend on the method capability, the dilution level (e.g., this criterion may be suitable for a two- or three-fold serial dilution assay format but not a 10-fold serial dilution format), and the intended use of the reported titer data in the clinical setting. If using the calculated mean titer approach, occasionally the mean titer may fall in between the dilutions because it is derived from observed values from multiple analyses. In this scenario, the ±1 dilution rule needs to be modified, and titers observed for a defined PC are rounded to the nearest dilution to yield the target titer.

Robustness and Reproducibility

Robustness and reproducibility of immunogenicity assays are discussed in ⟨1106⟩, which is in harmony with ⟨1225⟩ and ICH Q2(R1).

Robustness testing should be done as part of assay optimization during assay development, if at all possible. This is because it is unlikely that during validation an analyst would specifically attempt to make changes that might routinely occur (e.g., switching lots of materials or varying incubation times within certain limits). It is therefore necessary to understand which parameters of the method require strict adherence (e.g., concentration of coating antibody), with precise limits for the parameter delineated in the method, versus parameters that need less control and can have "approximate" descriptions in the method.

Robustness experiments can be performed in a simple fashion by varying one or two variables at a time or by DoE approaches, depending on how many factors will be tested at any one time and the extent to which interactions among parameters will be examined. The breadth of robustness testing depends on the intended use of the assay (e.g., a small study looking for gross changes in a toxicology experiment might need less robustness testing than some other studies). However, any assay used for clinical studies involving the registration of a drug should be examined thoroughly to ensure that it performs as expected on a routine basis over an extended period. Plate edge effect (or uniformity) should be examined during assay development. Other common factors that should be evaluated for impact include incubation times and temperatures, reagent concentrations, and cell densities.

If an assay needs to be transferred to a different laboratory, analysts should assess the reproducibility of the method in the new laboratory. This can be done as part of the assay transfer qualification and/or the validation of the method at the new facility. It is important to note that a properly designed and optimized assay, where critical parameters have been understood and controlled, should be robust enough to transfer without any issues. However, to ensure that the assay will perform in the same manner in each laboratory, several useful indicators can be assessed. Shared samples can be tested at both sites, and

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estimates of assay variability and quality control performance can be evaluated (see also the section *Transfers to Other Laboratories* in *Life Cycle Management*).

Stability

It is important to understand the optimal storage and handling conditions for assay samples, controls, materials, and reagents (see ⟨1106⟩ for additional guidance). For example, it may be important to provide advice to clinics on aliquoting procedures, time to freeze samples, storage temperature, and shipping conditions.

A key consideration for cell-based NAb assays is the stability of the cell line itself. Cell lines, as living entities, exhibit inherent variability and the potential to change over time or react to the environment in a way that can influence their response in the assay. For example, variations in levels of cell surface receptors may be affected by the number of cell doublings, time in culture, presence of certain media components (e.g., serum), and/or cell density. Therefore, during NAb assay development, cell line performance should be thoroughly characterized. Appropriate controls should be established for parameters including passage number, reagents, and media changes. An example is that the use of frozen cell aliquots of the same passage number for each assay can sometimes reduce variation because of changes in continuous culture.

Documentation of Pre-Study and In-Study Validation

The recommended documentation is described in chapter ⟨1106⟩ for both pre-study and in-study validation.

IN-STUDY VALIDATION ASSAY MONITORING

The ability of a NAb assay to perform in a reliable manner over time is important for conducting historical comparisons of NAb incidence for a single biotherapeutic as it advances through the clinical development life cycle. Also, verification that a validated assay continues to perform as expected is an ongoing process, and once the NAb assay is implemented, it is good scientific practice to monitor its performance by trending the results obtained with assay controls over time. It is recommended to use a statistical approach (e.g., Westgard rules) for assigning the threshold for assay failure and investigation based on behavior of the assay control (positive or negative). This may be easier to accomplish for highly precise assays than for assays that tend to be more variable. Regardless, it is recommended to tabulate the assay control values obtained from a minimum of 10 runs over an appropriate time period to establish a threshold and an approach for identifying assays that seem to be trending toward the assay limits. If the assay performs outside pre-established expectations, an investigation should be performed to identify the cause of the observation; implementation of appropriate step(s) may be necessary. If corrective step(s) are required, assay performance verification may be necessary to demonstrate that performance has returned to its original level. It is also important to identify variables that are likely to contribute to assay drift, such as the use of a new working cell bank, changes in lots of critical reagents, and different assay operators.

LIFE CYCLE MANAGEMENT

Changing the NAb Assay Format

During the drug development phase, it may be necessary to change the NAb assay format. For example, the assay format might be changed from cell-based to non-cell-based or vice versa to obtain an assay with better sensitivity or specificity, or other desirable characteristics. If NAb-related adverse events tend to be serious or life threatening (e.g., in the case of growth factor or cytokine drug products that have a nonredundant function), it is recommended to seek regulatory agency advice when considering a different assay format to ensure its suitability for detection of clinically relevant NAbs.

For all NAb assay format changes, an assessment of assay sensitivity and specificity is important. If the switch is being made for greater ease of performance without a significant improvement in assay characteristics, the ability of the previous and new formats to detect NAbs should be compared. This comparison should be made by using donor serum samples spiked with the PC antibody, as well as incurred study samples that have previously tested positive for NAbs. For the latter, a rate of concordance for sample results should be predetermined and met.

Transfers to Other Laboratories

NON-CLIA LABORATORIES

If the receiving laboratory is not certified under the Clinical Laboratory Improvement Amendments (CLIA) program, the capability of this lab to follow good laboratory practices (GLP) should be assessed before initiating any assay transfer activities. CLIA-certified laboratories are regulated by the Centers for Medicare and Medicaid Services of the U.S. Department of Health and Human Services. These regulations are defined in 24 CFR Part 493 and apply to laboratories testing human specimens for the purpose of disease diagnosis, prevention, monitoring, or treatment. For non-CLIA labs, the capability assessment may include evaluation of existing infrastructure for conducting cell-based or non-cell-based NAb assays, review of staff training records, and other activities. An informal feasibility study may be useful for assessing assay performance in the receiving lab. The transferring lab can simply provide the assay and reagents to the receiving lab, which can attempt a few runs and evaluate the assay controls. This exercise helps evaluate both the clarity of the written method and the receiving lab's ability to conduct the assay. The feasibility study results should be useful when determining the level of formal training that the transferring lab should provide to the receiving lab. Formal training, if needed, should adhere to a documented training plan. Assay controls

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and training samples prepared by spiking matrix samples with the PC NAb may be run by both the trainer and trainee following the same detailed method. The acceptance criteria for successful training should be clearly described in the training plan.

Formal assay transfer activities may commence after the receiving lab has been trained successfully and its equipment has been qualified. An assay transfer protocol should be written to describe the experiments that will be conducted during the assay transfer phase. The acceptance criteria for the experiments should be clearly defined. Some typical experiments that should be conducted by the receiving lab during assay transfer may include (a) running PC antibody curves, (b) testing matrix samples that have been left unspiked or have been spiked with PC NAb, and (c) testing different lots of critical reagents, performed by more than one analyst. For the experiments designated (b) and (c), it may be useful for both the transferring lab and receiving lab to conduct the experiments using the same training samples. Statistical analyses then should be performed on the data generated, to assess the degree of concordance between the two labs. The extent of concordance required for a successful assay transfer should be detailed in the protocol. In certain situations, it may be necessary to derive a new assay cut-point or a change in assay sensitivity because of a change in reagents or PC NAb. All changes to the method should be captured in a revised method supplemented by an assay transfer report that details all the experiments that support the changes. After a successful assay transfer, the receiving lab may implement the method for analyzing study samples. Appropriate assay trending approaches should be implemented to monitor assay performance and thereby ensure that it remains within recommended specifications.

CLIA LABORATORIES

For NAb assay transfers to a CLIA lab, one should follow the relevant guidelines for laboratory staff qualifications and for review and approval of the assay training and transfer documents by personnel who provide oversight to CLIA tests. The approach for NAb assay transfer to GLP (non-CLIA) labs, described above, may be used for CLIA lab transfers as well. Annual competency assessments, quality assurance monitoring and review of assay controls, reporting of individual patient test results to treating physicians, and proficiency testing are required of the CLIA-compliant laboratory. Proficiency testing (PT) should be performed twice annually. The testing may include preparation of pooled matrix samples that are tested to establish a baseline. Aliquots are then provided to the testing group in a blinded manner (at least 5 PT samples/PT event), and the results are compared to baseline. Overall, 4 of 5 samples must pass PT, and any discrepant results are investigated.

Cross-Validation and Bridging

Cross-validation may be necessary if the same assay needs to be run or maintained at multiple labs simultaneously. The first step is training of the personnel who run the assay at the new location; this is often provided by the originator lab. Several components of the assay transfer activities mentioned above may be applied during a cross-validation effort. Assay performance may be monitored using assay controls and matrix samples, which are spiked or left unspiked at both locations. If the NAb assay is altered within a study, bridging experiments should be performed. This may be accomplished by running incurred samples previously tested by the old method in the new method and assessing concordance. For a certain period of time, study samples may be run in both the old and new methods at the same time to ensure that the new method is acceptable.

Method Improvements or Changes

The laboratory should implement a process for introducing any known change in the assay that might impact assay performance. Appropriate qualification experiments should be conducted before introducing the change to ensure that it will not interfere with the historical performance of the assay. If the change requires an adjustment of the assay acceptance criteria, appropriate documentation may be required to show the impact of the adjustment on the intended use of the assay. A statistician should be consulted if needed.

For cell-based NAb assays, a change in the method might involve use of a different cell line or selection of a different assay endpoint with the same cell line to achieve improved assay characteristics. A change in the NAb assay readout platform is sometimes made (e.g., ELISA yielding absorbance values versus ECL values). In cases of method improvement, both assay development and assay validation will be needed.

CROSS-VALIDATION TO OTHER SPECIES

During the drug development cycle, there may be a need to change the assay matrix from one species to another (e.g., cynomolgus or chimp to human) or across patient populations. The change of assay matrix may require an investment in both assay development and assay validation efforts to ensure that the assay shows acceptable sensitivity, specificity, and other characteristics.

REAGENT REPLACEMENT

Reagent qualification is required if a critical reagent needs replacement using previously established qualification criteria. Ideally, the reference lot and the new lot should be compared by preparing assay controls. However, in many cases a reference lot may not be available for conducting a comparison. In this case, the new reagent should be tested and if the assay behaves as expected, it may be considered acceptable.

Assay Standardization

There is an emerging clinical need and apparent value in assay standardization (i.e., use of reference standards, platform, and reference method) to facilitate harmonization of the approach used for the detection of NAbs directed toward drugs in the

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same class of therapeutic [e.g., interferons, anti-tumor necrosis factor (TNF) MAbs, or erythropoiesis-stimulating agent therapies]. If several companies are involved, a consensus approach to standardizing the method should be undertaken. This requires agreement on a universal method and protocol with a common set of reagents in all laboratories concerned. For cell-based NAb assays, establishment and implementation of a common, master cell bank is critical. The reporting units for NAb-positive samples also must be unified, such as, expressing results as positive or negative versus specific values with units and/or using a standard protocol and common reagents for calculation of NAb activity. To confirm that standardization has been implemented successfully, the universal method should be used by all labs involved in the standardization effort to analyze a set of matrix samples spiked or unspiked with NAb PC in a blinded manner. Analysis of known positive and negative study samples also should be performed.

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⟨1111⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: ACCEPTANCE CRITERIA FOR PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage, and distribution of pharmaceutical preparations.

Microbial examination of nonsterile products is performed according to the methods given in the texts on *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Acceptance criteria for nonsterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts and molds count (TYMC) are given in *Tables 1* and *2*. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g., direct plating methods).

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- 10^1 cfu: maximum acceptable count = 20;
- 10^2 cfu: maximum acceptable count = 200;
- 10^3 cfu: maximum acceptable count = 2000; and so forth.

Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms

Route of Administration	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)	Specified Microorganism(s)
Nonaqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)
Rectal use	10^3	10^2	—
Oromucosal use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or1 mL)
Gingival use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)

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Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms *(continued)*

Route of Administration	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)	Specified Microorganism(s)
Cutaneous use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Nasal use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Auricular use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
			Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Candida albicans* (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10^2	10^1	Absence of *Staphylococcus aureus* (1 patch)
			Absence of *Pseudomonas aeruginosa* (1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulization)	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
			Absence of bile-tolerant Gram-negative bacteria (1 g or 1 mL)

Table 2. Acceptance Criteria for Microbiological Quality of Nonsterile Substances for Pharmaceutical Use

	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)
Substances for pharmaceutical use	10^3	10^2

Table 1 includes a list of specified microorganisms for which acceptance criteria are set. The list is not necessarily exhaustive, and for a given preparation it may be necessary to test for other microorganisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of microorganisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the microorganisms listed in *Table 1*, the significance of other microorganisms recovered should be evaluated in terms of the following:

- The use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract).
- The nature of the product: does the product support growth? does it have adequate antimicrobial preservation?
- The method of application.
- The intended recipient: risk may differ for neonates, infants, the debilitated.
- Use of immunosuppressive agents, corticosteroids.
- The presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

⟨1112⟩ APPLICATION OF WATER ACTIVITY DETERMINATION TO NONSTERILE PHARMACEUTICAL PRODUCTS

The determination of the water activity of nonsterile pharmaceutical dosage forms aids in the decisions relating to the following:

1. optimizing product formulations to improve antimicrobial effectiveness of preservative systems,
2. reducing the degradation of active pharmaceutical ingredients within product formulations susceptible to chemical hydrolysis,
3. reducing the susceptibility of formulations (especially liquids, ointments, lotions, and creams) to microbial contamination, and
4. providing a tool for the rationale for reducing the frequency of microbial limit testing and screening for objectionable microorganisms for product release and stability testing using methods contained in the general test chapter *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩.

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Reduced water activity (a_w) will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter.

Low water activity has traditionally been used to control microbial deterioration of foodstuffs. Examples where the available moisture is reduced are dried fruit, syrups, and pickled meats and vegetables. Low water activities make these materials self-preserved. Low water activity will also prevent microbial growth within pharmaceutical drug products. Other product attributes, for example, low or high pH, absence of nutrients, presence of surfactants, and addition of antimicrobial agents, as well as low water activity, help to prevent microbial growth. However, it should be noted that more resistant microorganisms, including spore-forming *Clostridium* spp., *Bacillus* spp., *Salmonella* spp. and filamentous fungi, although they may not proliferate in a drug product with a low water activity, may persist within the product.

When formulating an aqueous oral or topical dosage form, candidate formulations should be evaluated for water activity so that the drug product may be self-preserving, if possible. For example, small changes in the concentration of sodium chloride, sucrose, alcohol, propylene glycol, or glycerin in a formulation may result in the creation of a drug product with a lower water activity that can discourage the proliferation of microorganisms in the product. This is particularly valuable with a multiple-use product that may be contaminated by the user. Packaging studies should be conducted to test product stability and to determine that the container–closure system protects the product from moisture gains that would increase the water activity during storage.

Reduced microbial limits testing may be justified through risk assessment. This reduction in testing, when justified, may entail forgoing full microbial limits testing, implementing skip-lot testing, or eliminating routine testing.

Nonaqueous liquids or dry solid dosage forms will not support spore germination or microbial growth due to their low water activity. The frequency of their microbial monitoring can be determined by a review of the historic testing database of the product and the demonstrated effectiveness of microbial contamination control of the raw materials, ingredient water, manufacturing process, formulation, and packaging system. The testing history would include microbial monitoring during product development, scale-up, process validation, and routine testing of sufficient marketed product lots (e.g., up to 20 lots) to ensure that the product has little or no potential for microbial contamination. Because the water activity requirements for different Gram-reactive bacteria, bacterial spores, yeasts, and molds are well described in the literature,[1] the appropriate microbial limit testing program for products of differing water activities can be established. For example, Gram-negative bacteria including the specific objectionable microorganisms, *Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella* species will not proliferate or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not proliferate below 0.86, and *Aspergillus niger* will not proliferate below 0.77. Furthermore, even the most osmophilic yeast and xerophilic fungi will not proliferate below 0.60, and they cannot be isolated on compendial microbiological media.[1] The water activity requirements measured at 25° for the growth of a range of representative microorganisms are presented in *Table 1*.

Table 1. Water Activities (a_w) Required to Support the Growth of Representative Microorganisms

Bacteria	Water Activity (a_w)	Molds and Yeast	Water Activity (a_w)
Pseudomonas aeruginosa	0.97	*Rhyzopus nigricans*	0.93
Bacillus cereus	0.95	*Mucor plumbeus*	0.92
Clostridium botulinum, Type A	0.95	*Rhodotorula mucilaginosa*	0.92
Escherichia coli	0.95	*Saccharomyces cerevisiae*	0.90
Clostridium perfringens	0.95	*Paecilomyces variotti*	0.84
Lactobacillus viridescens	0.95	*Penicillium chrysogenum*	0.83
Salmonella spp.	0.95	*Aspergillus fumigatus*	0.82
Enterobacter aerogenes	0.94	*Penicillium glabrum*	0.81
Bacillus subtilis	0.90	*Aspergillus flavus*	0.78
Micrococcus lysodekticus	0.93	*Aspergillus niger*	0.77
Staphylococcus aureus	0.86	*Zygosachharomyces rouxii* (osmophilic yeast)	0.62
Halobacterium halobium (halophilic bacterium)	0.75	*Xeromyces bisporus* (xerophilic fungi)	0.61

Pharmaceutical drug products with water activities well below 0.75 (e.g., direct compression tablets, powder and liquid-filled capsules, nonaqueous liquid products, ointments, and rectal suppositories) would be excellent candidates for reduced microbial limit testing for product release and stability evaluation. This is especially true when pharmaceutical products are made from ingredients of good microbial quality, when manufacturing environments do not foster microbial contamination, when there are processes that inherently reduce the microbial content, when the formulation of the drug product has antimicrobial activity, and when manufacturing sites have an established testing history of low bioburden associated with their products. *Table 2* contains suggested microbial limit testing strategies for typical pharmaceutical and over-the-counter (OTC) drug products based on water activity. Other considerations, as listed above, would be applied when setting up the microbial limits testing program for individual drug products because water activity measurements cannot solely be used to justify the elimination of microbial content testing for product release.

[1] J. A. Troller, D. T. Bernard, and V. W. Scott. Measurement of Water Activity. In: *Compendium of Methods for the Microbiological Examination of Foods.* American Public Health Association, Washington, DC, 1984 pp.124–134.

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Similar arguments could be made for the microbial limits testing of pharmaceutical ingredients. However, this would require pharmaceutical manufacturers to have a comprehensive knowledge of the pharmaceutical ingredient manufacturer's manufacturing processes, quality programs, and testing record. This could be obtained through a supplier audit program.

Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity

Products	Water Activity (a_w)	Greatest Potential Contaminants	Testing Recommended
Nasal inhalant	0.99	Gram-negative bacteria	TAMC,* TCYMC, absence of *S. aureus* and *P. aeruginosa*
Hair shampoo	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of *S. aureus* and *P. aeruginosa*
Antacid	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of *E. coli* and *Salmonella* spp.
Topical cream	0.97	Gram-positive bacteria	TAMC, TCYMC, absence of *S. aureus* and *P. aeruginosa*
Oral liquid	0.90	Gram-positive bacteria and fungi	TAMC and TCYMC
Oral suspension	0.87	Fungi	TAMC and TCYMC
Topical ointment	0.55	None	Reduced testing
Lip balm	0.36	None	Reduced testing
Vaginal and rectal suppositories	0.30	None	Reduced testing
Compressed tablets	0.36	None	Reduced testing
Liquid-filled capsule	0.30	None	Reduced testing

* TAMC = Total aerobic microbial count; TCYMC = Total combined yeast and mold count.
[NOTE—The water activities cited in *Table 2* for the different dosage forms are representative, and companies are urged to test their individual products before developing a testing strategy.]

Water activity, a_w, is the ratio of vapor pressure of H_2O in product (P) to vapor pressure of pure H_2O (Po) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point or indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed.

The relationship between a_w and equilibrium relative humidity (ERH) is represented by the following equations:

$$a_w = P/Po \text{ and } ERH(\%) = a_w \times 100$$

The a_w measurement may be conducted using the dew point/chilled mirror method.[2] A polished, chilled mirror is used as the condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Sample preparation should be considered as it may affect the water activity level of the material tested. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations. These instruments are typically calibrated using saturated salt solutions at 25°, as listed in *Table 3*.

Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments

Saturated Salt Solutions	ERH (%)	a_w
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753
Magnesium nitrate [$Mg(NO_3)_2$]	52.9	0.529
Magnesium chloride ($MgCl_2$)	32.8	0.328

[2] AOAC International Official Method 978.18. In: *Official Methods of Analysis of AOAC International*, 17th edition, AOAC International, Gaithersburg, Maryland.

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⟨1113⟩ MICROBIAL CHARACTERIZATION, IDENTIFICATION, AND STRAIN TYPING

INTRODUCTION

Microorganisms, if detected in drug substances, excipients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished drug products, typically undergo characterization. This may include identification and strain typing, as appropriate. [NOTE—A *Glossary of Terms* is provided at the end of this chapter.] Routine characterization of microorganisms may include the determination of colony morphology, cellular morphology (rods, cocci, cell groupings, modes of sporulation, etc.), Gram reaction or other differential staining techniques, and certain key biochemical reactions (e.g., oxidase, catalase, and coagulase activity) that can be diagnostic. Microbial characterization to this level is sufficient for many risk-assessment purposes in nonsterile pharmaceutical manufacturing operations and in some sterile product manufacturing environments.

In some cases a more definitive identification of the microorganisms yields genus- and species-level identification. Beyond this, available methodologies can perform strain-level identification, which can be useful in an investigation to determine the source of the microorganism. Identification is especially common when organisms are recovered at atypically high rates or in numbers that exceed recommended levels for specific categories of products. Additionally, microbial identification is useful in aseptic processing and is necessary where sterility test positives have occurred and in the assessment of contamination recovered from failed aseptic process simulations, i.e., media fills.

Microbiological identification systems are based on different analytical methodologies, and limitations may be inherent to the method and/or arise from database limitations. Identification is accomplished by matching characteristics (genotypic and/or phenotypic) to an established standard (reference) organism such as a type strain. If a microorganism is not included in the database it will not be identified, so manufacturers should review the breadth of the database of the identification system they plan to use and its applicability to their needs. Users should consider which microbiological identification system(s) is (are) most applicable to their requirements. Bearing in mind both these limitations and the level of identification required (genus, species, strain), users also must select the appropriate technology to use in routine microbiological identification testing.

The need for microbial identification is specifically cited in USP general test chapter *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩. This chapter indicates a requirement for confirmatory identification tests for organisms that grow on selective or diagnostic media and demonstrate defined morphological characteristics. Also, USP general test chapter *Sterility Tests* ⟨71⟩ allows for invalidation of the test, if after identification of the microorganisms isolated from the test, the growth of this (or these) species may be unequivocally ascribed to faults with respect to the material and/or the technique used in conducting the sterility test procedure. USP general information chapter *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩ recommends that microbial isolates be identified at a rate sufficient to support the environmental monitoring program.

ISOLATION OF PURE CULTURES

The first step in identification is to isolate a pure culture for analysis. This is typically accomplished by successive streaking of the colony of interest in a quadrant pattern on appropriate general microbiological solid media with the objective of obtaining discreet colonies that usually yield pure cultures. This technique also allows phenotypic expression and growth of sufficient inoculum for succeeding identification procedures. Analysts should recognize that expression of the microbial phenotype (i.e., cell size and shape, sporulation, cellular composition, antigenicity, biochemical activity, and sensitivity to antimicrobial agents) may be affected by isolate origins, media selection, and growth conditions (see *Table 1*). Therefore, the preparatory media for identification and the number of subcultures may affect the results of phenotype identification methods.

Table 1. Phenotypic Characteristics Used in Microbial Taxonomy

Categories	Characteristics
Culture	Colony morphology, colony color, shape and size, pigment production
Morphological	Cellular morphology, cell size, cell shape, flagella type, reserve material, Gram reaction, spore and acid-fast staining, mode of sporulation
Physiological	Oxygen tolerance, pH range, temperature optimum and range, salinity tolerance
Biochemical	Carbon utilization, carbohydrate oxidation or fermentation, enzyme patterns
Inhibition	Bile salt-tolerance, antibiotic susceptibility, dye tolerance
Serological	Agglutination, fluorescent antibody
Chemo-taxonomic	Fatty acid profile, microbial toxins, whole cell composition
Ecological	Origin of the organism

In contrast, the microbial genotype generally is well conserved and unaffected by cultural conditions. Therefore, once the isolation of a pure, monoclonal colony is assured, the microorganism may be analyzed without concern over the most recent growth media or the viability of the isolate. *Table 2* lists genotypic characteristics that can be determined.

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Table 2. Genotypic/Phylogenetic Characteristics That Can Be Used in Microbial Taxonomy

Categories	Characteristics
Genotypic	DNA base ratio (G + C content), restriction fragment patterns, and DNA probes
Phylogenetic	DNA–DNA hybridization, and 16S and 23S rRNA sequences

Bacterial taxonomy as described in *Bergey's Manual of Systematic Bacteriology* (*Bergey's Manual*)[1] is at present accomplished by comparative analysis of genetic material. When the DNA from an unknown organism is compared to the DNA from a known organism, the degree of relatedness can be determined. Genotypic identification (*Table 2*) is accomplished through the use of DNA hybridization, restriction fragment pattern comparisons, and/or DNA probes. For example, greater than 70% relatedness with DNA–DNA hybridization indicates the organisms are the same species. Phylogenetic analysis (*Table 2*) is typically performed by comparing the base sequence of a portion of the 16S ribosomal RNA gene for bacteria, or the 23S ribosomal RNA gene for fungi. Polymerase chain reaction (PCR) is used to amplify these genes, and the amplified region is then isolated and base sequenced using an electrophoretic or dideoxy chain termination method. Comparisons can be made using validated proprietary databases or those that are publicly available. [**CAUTION**—Publicly available databases may not be validated.]

PRIMARY SCREENING AND CHARACTERIZATION

Microorganisms isolated on compendial media from samples of pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products may be physiologically stressed. The microorganisms will pass from a metabolic state suitable for survival under adverse ambient conditions to culture conditions that are far richer nutritionally and are at an optimal incubation temperature. This transition can be managed by careful handling of the isolates. In preparation for identification, individual representative colonies from the primary isolation media are streaked for monoclonal colonies onto solid media as described above. The first step is to determine the Gram reaction, cellular morphology, and in some cases diagnostic biochemical reactions of the bacteria isolates. This is a critical step for many phenotypic identification schemes. If the wrong characteristics are assigned to an isolate, subsequent testing may be conducted using the wrong microbial identification kit, resulting in an incorrect result. Several common preliminary screening tests are described below.

Gram Staining

Gram staining methods include the four-step method: crystal violet (primary stain), iodine (mordant), alcohol or alcohol–acetone (decolorizer), and safranin (counterstain). In the three-step method the decolorization and counterstaining steps are combined. Under optimal conditions, Gram-positive organisms retain the crystal violet stain and appear blue violet. Gram-negative organisms lose the crystal violet stain, so they contain only the counterstain safranin and appear red. Some bacteria may be Gram-variable. Common pitfalls in this method are that heat fixation may cause Gram-positive cells to stain Gram-negative, and older cultures may give Gram-variable reactions. Using too much decolorizer could result in a false Gram-negative result, and not using enough decolorizer may yield a false Gram-positive result. One variation that has advantages in some situations is to perform a methanol, rather than heat, fixation of the bacterial smear. In some cases alcohol fixation may give more consistent Gram stain results. In either method a Gram-positive and a Gram-negative control should be included to allow identification of errors in staining. Because the Gram-staining reaction must be read under a microscope, cellular morphology can be simultaneously ascertained.

Spore Staining

Spore staining can be accomplished using a malachite green stain for bacterial spores. A positive control should be included to allow identification of errors in spore staining.

Biochemical Screening

Key biochemical screening tests include (1) the oxidase test to separate Gram-negative, rod-shaped bacteria into nonfermenters (oxidase positive) and enteric (oxidase negative) bacteria, (2) the catalase test to separate *Staphylococci* (catalase positive) from *Streptococci* (catalase negative), and (3) the coagulase test to separate *Staphylococci* into coagulase negative (presumptively nonpathogenic) and coagulase positive (more likely pathogenic) *Staphylococci*.

For many types of investigations and routine surveying of manufacturing environmental bioburden, these few tests can provide sufficient information for ongoing evaluation. However, when circumstances dictate greater in-depth assessment, identification to the genus, species, or strain level can yield valuable insights about the nature and source of environmental bioburden. Also, microbial identification to the species and even strain level can be critical in assessing and mitigating risk from microbial contamination.

MICROBIAL IDENTIFICATION BY PHENOTYPIC METHODS

Phenotypic methods use expressed gene products to distinguish among different microorganisms. Generally, these require a relatively large number of cells in pure, monoclonal culture. Recovery and growth methods for microbial enumeration and

[1] *Bergey's Manual of Systematic Bacteriology, 2nd Edition,* 2003.

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identification are limited by the length of incubation and the fact that many organisms present in the environment are not recovered by general microbiological growth media. Additionally, freshly isolated, stressed microorganisms by subculture from primary recovery may not result in a full expression of phenotypic properties. However, methods based on carbon utilization and biochemical reaction, as well as fatty acid profiles by gas–liquid chromatography and whole-cell composition by MALDI–TOF mass spectrometry, are always based on inocula development for a specific identification system. These systems rely on specified culture media and incubation conditions to achieve consistent identification. Phenotypic microbial identification methods are successfully used in food, water, clinical, and pharmaceutical microbiological testing laboratories.[2] Phenotypic microbial identification methods provide information that enables microbiologists to make informed decisions regarding product risk and to recognize changes in environmental microflora. In many quality control investigations, phenotypic identification alone is sufficient and will enable scientists to conduct a thorough investigation and to recommend appropriate corrective actions as needed.

MICROBIAL IDENTIFICATION BY GENOTYPIC METHODS

Genotypic microbial identification methods are theoretically more reliable because nucleic acid sequences are highly conserved in most microbial species. Applicable genotypic methods include DNA–DNA hybridization, PCR, 16S and 23S rRNA sequencing, multilocus sequence typing (MLST), pyrosequencing, DNA probes, and analytical ribotyping. These methods can be technically challenging for microbiologists. They also require more expensive analytical equipment and supplies. Often these analyses are conducted by contract laboratories, government laboratories, universities, research institutes, or specialized laboratories within industrial firms. Therefore, the use of genotypic identification methods is typically limited to critical microbiological investigations such as product failure investigations. Further, if strain-level identification is done in the course of an investigation, analysts must ensure that the method is appropriate.

DNA sequencing of the first 500 base pairs of the 16S rRNA sequence is useful for identification to the species level but may not provide sufficient power to resolve among closely related species or strains of the same species. In contrast, Southern hybridization of restriction endonuclease digests is powerful and can be effective in demonstrating differences between two strains. If the banding patterns appear identical, this shows only that restriction endonuclease has similar cleavage sites in that region of the two organisms. Demonstration that the two organisms are the same should include two or more different restriction endonuclease digests, each of which yields bands in the area of interest. All bands from the two organisms must be identical.

In contrast to microbial identification, nucleic acid-based methods can be used to screen for specific microorganisms. The steps associated with this activity are sample collection, nucleic acid extraction, target amplification, hybridization, and detection. The problem of amplifying DNA from nonviable bacterial cells can be overcome by using reverse transcription to convert rRNA that is transitional, hence related to viability, to DNA for PCR amplification. Issues include the detection of microbial variants, limits of detection, matrix effects, positive cutoff verification, instrument and system carry-over, diagnostic accuracy, and reproducibility.

VERIFICATION OF MICROBIAL IDENTIFICATION METHODS

Microbial identification tests include serological tests, chemical reagents, reference organisms, and instrumentation. The verification of an identification test system can include one of the following: (1) using an existing system for parallel testing of microbial isolates obtained from routine testing (the number of isolates tested may be as high as 50, and any discrepancies in identification can be arbitrated using a referee method); (2) testing 12–15 known representative stock cultures of different commonly isolated species for a total of 50 tests; or (3) confirming that 20–50 organism identifications, including 15–20 different species, agree with the results of a reference laboratory testing of split sample.[3] In each case the appropriate quality control organisms, as recommended by the supplier and the compendia, should be included in the verification process.

With identification systems, verification of the identity of the species should be evaluated and the level of agreement should be considered. Typically greater than 90% agreement can be achieved with samples of microorganisms that are appropriate for the identification system. Groups of organisms that are challenging to identify (e.g., nonfermenting bacteria, corynebacteria, and coagulase-negative *Staphylococci*) may be included, when appropriate, in the verification process but may yield lower levels of agreement.

The hierarchy of microbial identification errors in descending order of impact is (1) misidentification to genera, (2) misidentification to species, and (3) no identification. Misidentification could lead to inappropriate corrective and preventive actions and product disposition.

A microbial identification system may not be able to identify an isolate because the organism is not included in the database, the system parameters are not sufficiently comprehensive to identify the organism, the isolate may be nonreactive in the system, or the species may not have been taxonomically described. Such isolates can be sent to the supplier of the microbial identification system for additional study and, if appropriate, added to the database. Alternatively, genotypic identification tests can be conducted, and the species can be added to an in-house database. Misidentification is more difficult to determine, but any microbial identification should be reviewed for reasonableness in terms of the microorganism's morphology, physiological requirements, and source of isolation. Organisms identified only to genus may be common for the numerous nonpathogenic species of *Staphylococcus, Corynebacterium* (and other small pleomorphic Gram-positive rods), and *Micrococcus*.

The most important verification tests are accuracy and reproducibility. These measurements can be defined as follows:

$$\text{Accuracy \%} = (\text{Number of correct results/Total number of results}) \times 100$$

[2] O'Hara, C.M., M.P. Weinstein, and J.M. Miller. Manual and automated systems for detection and identification of microorganisms. *ASM Manual of Clinical Microbiology, 8th Edition,* 2003.

[3] Cumitech 31. *Verification and Validation of Procedures in the Clinical Microbiology Laboratory.* Elder, B.L., S.A. Hansen, J.A. Kellogg, F.J. Marsik, and R.J. Zabransky, ASM, February 1997.

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Reproducibility % = (Number of correct results in agreement/Total number of results) × 100

The user should establish suitable acceptance criteria for accuracy and reproducibility, taking into account method capability.

Other measurements are sensitivity, specificity, and positive and negative predictive value. These measurements are best illustrated by an example. A clinical microbiology laboratory compared the frequency of isolation of a DNA hybridization probe to a culture method for the sexually transmitted bacterium *Neisseria gonorrhaeae.*[3] The frequency of isolation from clinical specimens was historically 10%. The lab ran 100 split samples, and the results are presented in *Table 3.*

Table 3. Comparison of the Distribution of Negative and Positive Results for the DNA Probe and Culture Methods

	Culture Results	
DNA Probe Results	Positive	Negative
Positive	9	2
Negative	1	88

Sensitivity = [9/(9 + 1)] × 100 = 90%
Specificity = [88/(88 + 2)] × 100 = 97.7%
Positive Predictive Value = [9/(9 + 2)] × 100 = 81.8%
Negative Predictive Value = [88/(88 + 1)] × 100 = 98.9%

Note that the positive predictive value (PPV) is not intrinsic to the test; it also depends on the prevalence of the microorganism in clinical samples. PPV is directly proportional to the prevalence of the disease or condition. In this example, if the group of people tested had included a higher proportion of people with infection, then the PPV would probably be higher and the negative predictive value (NPV) lower. If all persons in the group had infection, the PPV would be 100% and the NPV 0%. The mathematical derivation of these functions is outlined in *Table 4.*

Table 4. A Two-Row by Two-Column Contingency Table with Respect to the Reference Culture Method and the Alternate PCR Method (After ISO 5725-1 and 5725-2 2004)*

	PCR		
Culture	**Positive**	**Negative**	**Sum**
Positive	a True Positive	b False Negative	a + b
Negative	c False Positive	d True Negative	c + d
Sum	a + c	b + d	

* ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions and ISO 5725-2:1994 Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic methods for the determination of repeatability and reproducibility of standard measurement methods.

Inclusivity (%) = [a/(a + b)] × 100
Exclusivity (%) = [d/(c + d)] × 100
Positive Predictivity (%) = [a/(a + c)] × 100
Negative Predictivity (%) = [d/(b + d)] × 100
Analytical Accuracy (%) = [(a + d)/(a + b + c + d)] × 100
Kappa Index = 2(ad − bc)/[(a + c) × (c + d) + (a + b) × (b + d)]

Phylogenetic Considerations

The second edition of *Bergey's Manual* represented a major departure from the first edition, and also from the eighth and ninth editions of the *Manual of Determinative Bacteriology.* The organization of content in *Bergey's Manual* follows a phylogenetic framework, based on analysis of the nucleotide sequence of the ribosomal small subunit 16S RNA, rather than a phenotypic structure.

Phylogenetic trees or dendrograms show the closest genetically related organisms. The application of this technology has resulted in taxonomic revisions and the renaming of some well-known microorganisms; e.g., the fungus *A. niger* ATCC 16404 was renamed *A. brasiliensis.* In general, organisms with relatedness less than or equal to 97% are considered different genera and those with relatedness less than or equal to 99% are considered different species,[4] but there are many exceptions to this generalization.

Differences in genotype and phenotype are relatively uncommon, e.g., same or very similar genotype shared by microorganisms with different phenotypes, similar phenotypes but different genotypes, and microorganisms that are genotypically too distant to be the same species or genus. The concept of polyphasic taxonomy[5] that refers to assembly and use of many levels of information, e.g., microbial characterization, phenotypic and genotypic data, and origin of the

[4] J.E. Clarridge III. The Impact of 16S rRNA Gene Sequencing Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases, *Clin. Microbiol. Rev. 17 (2)* 840–862, 2004.
[5] Gillis, M., P. Vandamme, P. De Vos, J. Swings and K. Kersters. Polyphasic Taxonomy. *Bergey's Manual of Systematic Bacteriology, 2nd Edition,* 2003.

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microorganisms, can be successfully applied to microbial identification. This avoids decisions made solely using genotypic data that make no sense when the microbial characteristics, testing history, and source of isolation are considered.

GLOSSARY

Microbial classification: The arrangement of microorganisms into taxonomic groups based on their similarities and relationships.

Microbial identification: The determination of which broad group (e.g., bacteria, yeast, or mold) or narrow group (e.g., genus and/or species) to which a laboratory isolate belongs.

Microbial characterization: The use of colony growth, cellular morphology, differential staining, and key diagnostic features to characterize a laboratory isolate for trending and investigative purposes without identification, e.g,. nonpathogenic Staphylococci.

Mol %GC: The molecular percentage of guanine–cytosine range within the chromosomal DNA. [NOTE—The %GC + %AT = 100%.]

Phylogenetic species: A species consisting of many strains including the type of strain that shares at least 70% total genome DNA–DNA hybridization and less than 5° ΔTm (difference in melting point of the hybrid).

Polyphasic taxonomy: Taxonomy that assembles and assimilates many levels of information from molecular, physiological, morphological, serological, or ecological sources to classify a microorganism.

Relatedness: The extent of relationship or similarity of two (or more) organisms on a Phylogenetic Tree or a Dendrogram.

rRNA Sequence: The DNA sequences that encode rRNA used in protein synthesis are highly conserved among microorganisms of a common ancestry. They are used to determine the phylogenetic distance between organisms and are useful in microbial taxonomy and identification.

Strain: A specific isolate of a species that is maintained in pure culture and is characterized. The type strain is representative of the species that provides a reference for the species based on its historic isolation, characterization, and deposition in recognized culture collections.

Strain typing: Strain typing is an integral part of epidemiological investigations in clinical and public health microbiology. Methods including pulsed-field gel electrophoresis, riboprinting, arbitrarily primed polymerized chain reaction, and whole genome ordered restriction or optical mapping can be used to demonstrate that microbial species are the same strain and most likely are from a common source.

⟨1115⟩ BIOBURDEN CONTROL OF NONSTERILE DRUG SUBSTANCES AND PRODUCTS

INTRODUCTION

In terms of microbiological contamination risk control, there are two broad categories of drug products: (a) sterile products, in which the bioburden is essentially eliminated using validated methodologies, and (b) nonsterile products for which the final product bioburden is controlled to appropriate levels based on product attributes, route of administration, and target patient population.

Microbial content in nonsterile products is controlled to a level consistent with patient safety. Use of excessive controls that would add complexity or cost without a commensurate safety benefit is not advantageous in terms of added value to either the patient or the manufacturer. Therefore, a pragmatic scientific approach to management of the microbial bioburden in nonsterile products requires consideration of patient risk and contamination control objectives to achieve a practical and appropriate level of risk management.

A critical consideration in ensuring product quality is to prevent conditions within the manufacturing facility or manufacturing process that favor the proliferation or ingress of microorganisms. Microbial growth in excipients, components, and drug substances is a concern because it creates the possibility that viable microbial content could reach unacceptable levels. Bioburden levels in the ranges of those recommended in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ are recognized as safe and do not pose risk of infection or microbial toxins. Manufacturers should have a clear understanding of situations that could favor microbial growth within their facilities and materials and should implement practical countermeasures.

This chapter outlines a risk-based approach to the control of contamination in nonsterile product manufacturing. The manufacture of nonsterile products and the management of their microbiological content are distinctly different from those required for sterile products. Sterile products are administered parenterally by means of injection or are applied topically to sensitive tissues where the risk of infection is comparatively high.

In contrast, nonsterile products are administered to regions of the human body that are rich in microbial flora and have physical or immunological barriers to infection. Examples include the oral cavity, skin, nasopharynx, vagina, and rectum, which harbor a high and diverse viable microbial population. Recent findings from the human microbiome project (*1*) underscore the enormous size and diversity of bacterial populations associated with humans. A healthy adult has a bacterial population that averages approximately 10^{14} bacteria, a number that exceeds the individual's own cells by a factor of 10. All humans carry on or in their bodies microorganisms that under certain conditions may cause infection in other humans. Because these microorganisms are present on humans means they may be present transiently in the nonsterile manufacturing environment. Their ubiquity in nature and infrequent association with infection confirms that the risk associated with them is very low. However, good hygienic controls and selection of suitable gowning systems are important considerations for products that are intended for patients who may be immunocompromised.

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The following list provides a hierarchy of broad categories of nonsterile pharmaceutical products with respect to potential risk of microbiological contamination (from high to low) (*2*):

- metered-dose and dry powder inhalants
- nasal sprays
- otics
- vaginal suppositories
- topicals
- rectal suppositories
- oral liquids (aqueous)
- liquid-filled capsules
- oral tablets and powder-filled capsules

When formulators evaluate the susceptibility of nonsterile pharmaceutical products to microbial hazards, considerations include whether the active ingredient has inherent antimicrobial activity, the microbiological content of excipients, inclusion of antimicrobial preservatives in the formulation, and water activity. In addition, manufacturers should consider whether processing steps and hold periods could result in changes in the bioburden.

Nonsterile products are expected to have some bioburden, which should be controlled within a suitable range (see ⟨1111⟩). The risk of infection resulting from a nonsterile drug product generally is low, regardless of the route of administration, provided appropriate precautions and procedures are followed. General chapters *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩ and *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩ provide methods, and ⟨1111⟩ provides information for the evaluation of microorganisms isolated during nonsterile drug product manufacturing. It is not possible to provide a comprehensive product-by-product list of objectionable microorganisms. The degree to which any organism may be objectionable depends on the product attributes, route of administration, and patient population. Manufacturers are responsible for determining whether microorganisms recovered from drug products are objectionable. In general, objectionable microorganisms are those that are known to be truly pathogenic, considering the product's route of administration. Guidance for risk factors to be considered is also provided as a bulleted list in ⟨1111⟩. Risk may also arise where microorganisms are able to proliferate in sufficient numbers that could result in an unacceptable level of patient risk to a level above the ranges recommended in ⟨1111⟩. Microbiological risk should be assessed on a case-by-case basis during the development of a new product and should be evaluated during the validation of the manufacturing process.

The proliferation of microbial contamination in a production facility, in products, or in product ingredients is an objectionable condition. Microbial proliferation within a facility creates conditions that favor the spread of contaminants, potentially in dangerous numbers, into ingredients, primary packaging materials, and even into product itself. Proliferation of microorganisms in ingredients or production intermediates must be prevented because it creates a risk due to microbial toxin and could result in damage to the chemical and pharmacological properties of the drug.

U.S. REGULATORY GUIDANCE DOCUMENTS

The U.S. Code of Federal Regulations includes the Food and Drug Administration's Good Manufacturing Practice (GMP) requirements, which are found in Part 211 (*3*). These regulations contain general requirements for the manufacture of pharmaceutical products. Pertinent sections of 21 CFR 211 include: *211.42 Design and construction*—requires that buildings used for pharmaceutical manufacturing and associated activities are of suitable size, construction, and location; *211.46 Ventilation, air filtration, air heating and cooling*—requires adequate equipment to control microorganisms, dust, humidity, and temperature and differential air pressures be provided when appropriate for drug product manufacturing; *211.56 Sanitation*—requires written procedures for use of suitable rodenticides, insecticides, fungicides, fumigating agents, and cleaning and sanitizing agents, and written procedures shall be designed to prevent the contamination of equipment, components, drug product containers, closures, packaging, and labeling materials; *211.82 Receipt and storage of untested components, drug product containers, and closures*—requires an acceptance, quarantine, testing, and release procedure for components and containers; *211.110 Sampling and testing of in-process materials and drug products*—requires testing of in-process manufacturing controls that will include bioburden determination. Objectionable organism considerations: *21 CFR 211.84(d)(6)*—requires microbiological testing of incoming containers and components with potential for microbiological contamination that is objectionable in view of their intended use; *21 CFR 211.113(a)*—requires written procedures directed to control of objectionable microorganisms in drug products not required to be sterile; *21 CFR 211.165(b)*— requires appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms.

MICROBIAL CONTROL CONSIDERATIONS DURING PRODUCT DEVELOPMENT

A formal risk assessment program that identifies risk modalities and assigns critical control points for manufacture of nonsterile product is useful. Hazard Analysis and Critical Control Point programs (*4*) are widely used to assess and mitigate microbial risk in food manufacturing and may also be useful for manufacturers of nonsterile drug products. Points to be considered by pharmaceutical microbiologists when they assess the potential risk associated with nonsterile drug product manufacture are listed below:

- synthesis, isolation, and final purification of the drug substance
- microbiological attributes of the drug substance
- microbiological attributes of excipients and intermediates
- formulation and chemical and physical attributes of the drug product
- antimicrobial properties of the material, e.g., water activity or others

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- manufacturing process
- delivery system
- packaging
- storage conditions for intermediates and the finished dosage form
- route of administration
- expected treatment procedure and dosage regimen
- population to which the product is delivered (e.g., neonates, immunocompromised patients, etc.)

Thorough consideration of these factors is valuable in defining appropriate manufacturing facility requirements and process control point measures that limit conditions that favor microbial proliferation and/or ingress.

MICROBIAL CONTROL CONSIDERATIONS DURING MANUFACTURING

Although many factors (*Figure 1*) can result in the introduction of microorganisms, some of these are more likely to result in microbial contamination. These manufacturing factors are, in descending order (*5*): (1) ingredient water, (2) pharmaceutical ingredients, (3) process equipment, (4) manufacturing personnel, and (5) manufacturing environment.

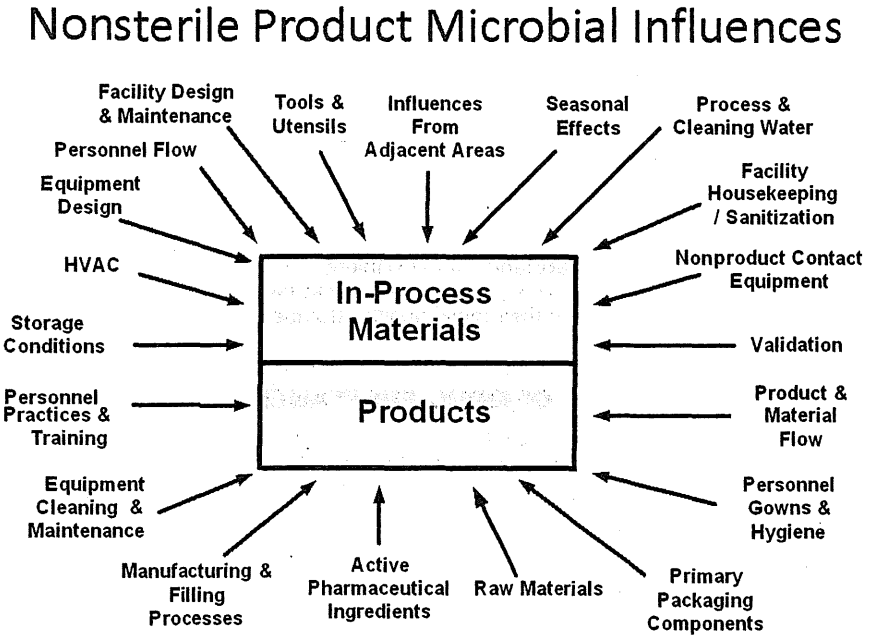

Figure 1. Factors that contribute to nonsterile product bioburden.

Water Systems and Use

Water used in active ingredient manufacturing, formulation, cleaning, and housekeeping is the single most important risk element contributing to the contamination of nonsterile products. The quality or type of water used for nonsterile product formulation and final rinse of clean equipment should be chosen based on product risk. Purified waters used in pharmaceutical manufacturing are deionized and thus do not contain chlorine to control microbial growth. Substantial populations of Gram-negative rod-shaped bacteria and some molds are able to grow in such purified dechlorinated water, particularly in holding tanks at or around ambient temperatures. Standing water should be drained or physically removed quickly and efficiently from production vessels and equipment, as well as work surfaces and floors. Chlorinated potable water (city water) may be appropriate for some cleaning, housekeeping, and sanitization activities. Water quality for processing application uses should be determined on a case-by-case basis. Additional guidance for water system design and operation can be found in *Water for Pharmaceutical Purposes* ⟨1231⟩.

Process waters used for manufacturing of excipients, and, in some cases, active ingredients for nonsterile products present a substantial risk for microbial colonization and proliferation, particularly for ingredients of natural origin that have received minimal processing to reduce bioburden or to control microbial proliferation. Formulating and manufacturing equipment can be a source of contamination, and risks are higher when water and ingredients that are susceptible to microbial survival or growth are used. Therefore, cleaning, drying, and, where appropriate, sanitization of manufacturing equipment can be beneficial, but disinfectant residues should be limited in the operating environment and should be removed from product-contact surfaces. The isolation of waterborne organisms, particularly Gram-negative rods, is a likely indicator of failure to remove standing water on equipment and environmental surfaces.

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Active Pharmaceutical Ingredients, In-Process Materials, and Excipients

Ingredients and excipients used in nonsterile product manufacturing processes are important sources of microbiological contamination. Vendor evaluation, specifications, testing, package selection, shipping, storage conditions, expiry dates, and assessment for likely contamination or proliferation risk are all critically important in the reduction of microbial risk associated with these materials. Of particular concern are unprocessed materials of natural origin, those that have high water activity (see *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* ⟨1112⟩ for additional information), synthetic processes with aqueous isolation steps or open processing, and biological processes with limited downstream purification or lacking defined microbial removal step(s). In-process bioburden monitoring points should be established at points in the process that are either immediately before or immediately follow a potential bioburden reduction process (such as an organic solvent extraction, heating, large change in pH) and/or immediately prior to final fill as determined by the risk analysis performed as suggested in the section *Microbial Control Considerations during Product Development*.

Manufacturers should sample incoming materials and should ensure proper contamination-control conditions for weighing and material addition to process equipment. All sampling and weighing equipment should be properly cleaned, sanitized, stored, and identified. The activities and the associated controls implemented to prevent microbial colonization and proliferation should be based on a documented, prospective risk-assessment and contamination-control strategy.

Pharmaceutical ingredients of consistently suitable microbial quality are an important element of the microbiological control program for nonsterile products. Procurement of ingredients of appropriate microbial quality requires the identification of vendors with the demonstrated capacity to produce drug substances or excipients of suitable quality. Supplier survey assessments may be conducted periodically to establish that the supplier has a well-designed and maintained microbiological control program for its manufacturing and primary packaging facilities. Materials that have low water activity, possess high or low pH, are not of natural origin, are inherently antimicrobial, or contain an antimicrobial preservative are generally not at risk for microbial proliferation. Risk assessments should consider ingredient characteristics regarding microbial survival, support of microbial growth, or frank antagonism to microbial survival.

The introduction of moisture into stored materials notably increases the risk of microbial contamination. Condensation in storage tank headspace or impermeable storage containers can result in contamination of materials with waterborne organisms or fungi even when the product under storage is expected to preclude microbial colonization or proliferation.

Microbiological examination of pharmaceutical ingredients (see chapters ⟨61⟩, ⟨62⟩, and ⟨1111⟩) can provide important insights into the microbiological quality of drug substances and excipients.

Primary packaging and intermediate containers (e.g., drum liners, plastic bags, and so on) can be a source of microbial contamination, and manufacturers should consider their initial quality, storage conditions, preparation, and handling procedures.

MICROBIAL CONTROL OF DRUG SUBSTANCE MANUFACTURING

The approaches to microbial control described in this chapter can be applied with minimal change to drug substances manufactured either by biological processes or organic synthesis. The processes in organic synthesis often use extremes of temperature, pressure, pH, and other conditions that inhibit or actively destroy microorganisms. When the chemical process for manufacturing a drug substance is complex, the final steps often have the greatest potential effect on the bioburden. As is the case for drug product manufacturing, the water used in the process presents the greatest challenge to microbial control. The types of water used in these processes vary. Potable water typically is used in early-stage processing and cleaning, and Purified Water and Water for Injection often are used for downstream processing and cleaning. Use of Water for Injection is typically restricted to the final steps of bulk chemical or biological drug substance manufacturing that will ultimately be used in the formulation of sterile products. These processes are frequently performed almost entirely within closed vessels, which minimizes external facility considerations. Some process equipment is suitable for clean-in-place operations that use agents that are not only effective cleansers but also in some cases inhibit microbial growth/proliferation. The materials used in drug substance manufacturing include natural substances, organic compounds of various types, inorganic salts, acids, bases, and organic solvents, and the potential for microbial contamination from these materials clearly is variable. Personnel interventions in these processes are limited by the nature of the equipment and consideration for worker safety, and in biologics processing and certain late stages of organic synthesis the manufacturing environments may be classified (e.g., ISO 7 and ISO 8). Limiting the involvement of personnel and use of controlled environments both reduce opportunities for microbiological contamination.

Equipment Design and Use

When possible, specifications for the selection of equipment that will be used in the manufacture of nonsterile products should include sanitary design. Equipment and utensils should be cleanable so contaminants and residual products can be reliably removed. Equipment should use sanitary fittings and should be designed for easy use of cleaning and sanitizing agents and complete rinse water drainage. Residual water in tanks, piping, or on equipment surfaces introduces the risk of colonization by waterborne organisms. Manufacturing equipment that cannot be cleaned in place should be readily accessible for manual cleaning, and parts that must be cleaned out of place should be not only easily accessible but also readily or easily removable. A further consideration is the compatibility of equipment with the typical range of disinfectants, including sporicides, used in cleaning procedures to sanitize equipment.

The preferred material of construction for equipment and utensils that will be in contact with product is austenitic stainless steel. Manufacturers should consider the surface finish on product-contact materials, for which a roughness average (RA) of 15–20 µm is a reasonable rule of thumb. Surfaces for general equipment and machine surfaces need not be polished beyond these RA values. Other materials of construction should be nonporous, smooth, and compatible with the products and cleaning materials. Process piping systems also should follow sanitary design principles and should be sloped to facilitate drainage, and the equipment should contain flush gaskets to prevent material build-up and to facilitate cleaning. The 3A Sanitary Standards

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(see www.3-A.org) provide generally useful guidance for process layout and design and machinery selection. Equipment cleaning procedures should be detailed and should ensure that the equipment is completely dry after cleaning and is stored in a manner that prevents microbial proliferation. Manufacturers should implement procedures for the protection of cleaned equipment and utensils before their next use. Cleaning and sanitization process validation should include the evaluation of microbial content both after sanitization and before use. Properly designed storage protection should mitigate the possibility of microbial growth before use, so after proper storage conditions are validated ongoing monitoring of equipment and utensils should not be required. Surface microbial sampling either immediately after cleaning or immediately before use must be done with caution; media residues and residual moisture must be carefully eliminated if sampling is performed.

Manufacturers should evaluate whether products that are manufactured using a piece of processing equipment may, under some processing circumstances, promote the growth of microorganisms. This evaluation is necessary to properly establish processing hold times and to define equipment use conditions following cleaning. The use of sanitizing agents on product-contact surfaces is not required when the cleaning procedures that remove chemical residues also remove microorganisms. In addition, there is a risk of product contamination with sanitization agents. With a validated cleaning process, the absence of chemical residues and visible standing water may provide assurance of the cleanliness of the equipment without routine chemical disinfection and also may obviate the need for microbiological monitoring of the equipment.

Personnel

In addition to maintaining personal hygiene, operators should be trained and dressed appropriately for the function they are performing (*Table 1*).

Table 1. Gowning in Manufacturing Areas

Protective Clothing	Operators in Formulation and Primary Packaging Areas
Plant uniform or plant uniform with overall for high-risk product and environment	Yes
Hair/beard covering	Yes
Safety glasses	Yes
Dedicated shoes or shoe coverings	Yes
Gloves	Yes (if in direct product contact)
Face masks	Yes (if in direct product contact)

The Manufacturing Environment

As noted, the environmental risks and controls for nonsterile products are different from those for aseptically manufactured sterile products. Unlike aseptic processing for which facility requirements are generally uniform in specification and performance, nonsterile product manufacturing environments typically involve diverse products and microbial contamination control requirements. In general, liquid, cream, or ointment products require a greater level of contamination risk mitigation than do solid dosage forms.

Common design elements to control microbial contamination may include the following:

- Walls, ceilings, and floors are constructed of nonporous materials that are readily cleanable and are resistant to cleaning agents and disinfectants.
- Floor drains are permitted in nonsterile product manufacturing areas provided that they can be closed during processing or fitted with a suitable air break if they are open during area and equipment cleaning.
- Access should be limited to essential personnel.
- Material, equipment, and personnel flows should avoid contamination.
- Ventilation and air filtration should be adequate to maintain the specified cleanliness, space pressurization (if required), temperature, and relative humidity.
- Good housekeeping and good general hygiene should be applied at all times.
- Cleaning and use status of all tools and implements used in production and all process equipment should be known at all times.
- Product-contact or water-supply tubing, valves, and fittings should be cleaned and sanitized according to a defined schedule, should be stored dry, and should be labeled with respect to status.
- Manufacturers should implement a formal housekeeping and sanitization program for operating areas, corridors, equipment storage, material staging, and other common areas.

Classified environments are not required for nonsterile product manufacturing, e.g., those specified in ISO 14644-1 (*6*). ISPE Baseline Guide No. 2, Oral Solid Dosage Forms (*7*) provides minimal acceptable design features for facilities for manufacturing nonsterile products.

MICROBIAL ASSESSMENT OF NONSTERILE PRODUCT MANUFACTURING ENVIRONMENTS

As described above, microbiological monitoring of the manufacturing environment can serve as an adjunct to control and generally is a qualitative assessment tool in a properly implemented formal risk-based microbiological control program. A

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monitoring program commensurate with the product bioburden risk can help confirm the effectiveness of microbiological controls and may facilitate early detection of potential problems. Microbial methods and practices for aseptic facilities may be used, but the contamination recovery rates defined in *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩ are not intended for nonsterile environments. Classified environments of the same class do not necessarily have similar microbiological control capabilities. Because the majority of organisms recovered in environmental monitoring are of human origin, the levels of transient contamination recovered depend largely on the level of human activity and gowning requirements. Gowning requirements are not as extensive in nonsterile product manufacturing as they are in aseptic processing, and thus in nonsterile environments manufacturers can monitor bioburden with reduced frequency and with expectations of higher bioburden recovery. Periodic assessments of production plant hygiene can offer useful insights into the effectiveness of the facility's cleaning and environmental controls.

During nonsterile product manufacturing, microbiological monitoring of the environment need not be as rigorous as that required during aseptic processing. Similarly, during nonsterile product manufacturing microbial risk mitigation is different from that for sterile products. In nonsterile products, manufacturers can expect intrinsic microbial bioburden that, properly controlled, does not result in risk to the end user. Manufacturers should establish acceptable levels of microorganisms within each product (*3*) and should sufficiently identify microorganisms to gain an appropriate understanding of bioburden patterns and seasonal variability.

Microbial Sampling

During nonsterile product manufacturing, the air sampling methods used for environmental monitoring are active or passive. Active devices sample appropriate air volumes and deposit viable organisms on solid media plates or strips. Results typically are expressed as colony-forming units per unit volume although alternate methods may be used in lieu of recovery and growth on media. Passive sampling in the form of settling plates can be used in lieu of active air samplers.

Personnel monitoring typically is not required in nonsterile product manufacturing except when near-aseptic gowning materials are employed.

Sampling locations should be selected based on risk evaluation followed by a general hygienic survey of the environment. High-traffic areas where materials are transferred into and out of the area may be particularly prone to transient microbial contamination. Microbiological monitoring is not required in areas beyond the point where product has been placed into primary packaging containers.

Alternate nongrowth-based microbiological methods can be substituted for growth-based methods at the option of the user. Because there are no standardized environmental sampling methods and because monitoring is intended as a qualitative evaluation of general facility hygiene, there is no need for comparative studies between growth- and nongrowth-based methods. A small number of parallel samples is sufficient to establish a comparative baseline for an environment.

The frequency of monitoring should reflect the potential risk associated with the dosage form (see *Introduction*). Additionally, some products may have innate antimicrobial activity because of their attributes such as low water activity or inclusion of an antimicrobial preservative or an active ingredient that is itself an antimicrobial agent, e.g., an antibiotic or antitumor agent. Products that are resistant to microbial colonization or have microbiocidal or microbiostatic characteristics require little or no microbiological monitoring.

In general, environments for tablet and powder- and liquid-filled capsule manufacturing should require no monitoring or infrequent monitoring. Monitoring programs should be risk based, and the frequency and number of sampling sites should reflect the risk level. Manufacturing areas for higher-risk dosage forms such as inhalant products require more frequent monitoring and typically are manufactured in rooms classified to a particulate air quality level, e.g., ISO 8.

For most nonsterile product manufacturing environments, because of their limited environmental controls and comparatively low product risk, the establishment of alert and action levels may not be required. Environmental monitoring is considered an informational survey of the general hygienic conditions of the environment and should not be used in product-release decisions. Monitoring of unclassified environments is not required.

General information chapter *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩ contains general information about microbial characterization. In most nonsterile hygienic assessments, characterization of the microorganisms, cellular morphology, Gram reaction, and simple diagnostic testing are sufficient.

Active Measures for Microbial Control

In addition to facility and process design considerations and equipment cleaning and storage controls, there are instances in which active means for addressing the contamination risk are required. The microbiological control of nonsterile products can be enhanced by the adoption of direct contamination control processes such as the following:

- decontamination of product contact surfaces, materials, and containers, typically by means of heat treatment (in the most critical applications sterilization can be used)
- chemical or physical (e.g., dry or moist heat) bioburden reduction treatments for raw materials and active ingredients
- use of closed, cleaned or decontaminated systems for handling and transfer of materials
- use of disposable components or utensils
- improved gowning materials for operational personnel
- use of classified environments in high-risk operations

These measures can be applied as required to improve the microbial contamination control during the manufacture of nonsterile products.

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OVERALL MANAGEMENT OF A MICROBIOLOGICAL CONTROL PROGRAM

The management of a successful microbiological control program includes the following: identification of suitable suppliers of pharmaceutical ingredients and excipients that have good microbiological quality; conducting a microbial risk assessment of the manufacturing process and packaging system; and the establishment of an appropriate monitoring and control system.

Although environmental contamination is by no means the most significant cause of nonsterile product recalls or contamination events, environmental monitoring may be a program adjunct to the microbiological control program. Microbial monitoring is an assessment and is not by itself a contamination control activity. There have been no scientifically controlled studies demonstrating what linkage, if any, exists between airborne or surface monitoring results and microbiological safety of the final product.

The microbiological contamination control program should be developed for identifying and controlling product risk, based on a formal assessment of risk modalities. The risk analysis should result in the identification of critical control points and should facilitate proper equipment selection, process layout and design, and facility design requirements.

Critical factors for the prevention of microbiological contamination during nonsterile product manufacturing are the control of the microbiological quality of ingredients and water, along with the development of proper cleaning and sanitization procedures. Microbiological monitoring does not mitigate risk, but it may serve as a sentinel.

No monitoring program can provide the assurance of contamination control as effectively as sound, proactive and preventive measures. Consistent control of contamination can be achieved mainly by an overall process evaluation assessing each of the control elements described above via risk assessment. Risk assessment may be coupled with active evaluation studies to ensure that appropriate measures are in place to prevent conditions conducive to contamination.

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⟨1116⟩ MICROBIOLOGICAL CONTROL AND MONITORING OF ASEPTIC PROCESSING ENVIRONMENTS

Microbiologically controlled environments are used for a variety of purposes within the healthcare industry. This general information chapter provides information and recommendations for environments where the risk of microbial contamination is controlled through aseptic processing. Products manufactured in such environments include pharmaceutical sterile products, bulk sterile drug substances, sterile intermediates, excipients, and, in certain cases, medical devices. Aseptic processing environments are far more critical in terms of patient risk than controlled environments used for other manufacturing operations —for example, equipment and component preparation, limited bioburden control of nonsterile products, and processing of terminally sterilized products. In this chapter, the type of aseptic processing is differentiated by the presence or absence of human operators. An advanced aseptic process is one in which direct intervention with open product containers or exposed product contact surfaces by operators wearing conventional cleanroom garments is not required and never permitted. [NOTE—A description of terms used in this chapter can be found in the *Glossary* at the end of the chapter.]

The guidance provided in this chapter and the monitoring parameters given for microbiological evaluation should be applied only to clean rooms, restricted-access barrier systems (RABS), and isolators used for aseptic processing. ISO-classified environments used for other purposes are not required to meet the levels of contamination control required for aseptically produced sterile products. The environments used for nonsterile applications require different microbial control strategies.

A large proportion of products labeled as sterile are manufactured by aseptic processing rather than terminal sterilization. Because aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention of microorganisms from entering open containers during processing, product bioburden as well as the bioburden of the manufacturing environment are important factors governing the risk of unacceptable microbial contamination. The terms *aseptic* and *sterile* are not synonymous. *Sterile* means having a complete absence of viable microorganisms or organisms that have the potential to reproduce. In the purest microbiological sense, an *aseptic* process is one that prevents contamination by the exclusion of microorganisms. In contemporary aseptic healthcare-product manufacturing, *aseptic* describes the process for

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handling sterilized materials in a controlled environment designed to maintain microbial contamination at levels known to present minimal risk.

In any environment where human operators are present, microbial contamination at some level is inevitable. Even the most cautious clean-room environment design and operation will not eliminate the shedding of microorganisms if human operators are present. Thus, an expectation of zero contamination at all locations during every aseptic processing operation is technically not possible and thus is unrealistic. There are no means to demonstrate that an aseptic processing environment and the product-contact surfaces within that environment are sterile. Monitoring locations should be determined based upon a assessment of risk. Although manufacturers should review environmental monitoring results frequently to ensure that the facility operates in a validated state of control, monitoring results can neither prove nor disprove sterility. Because of the limitations of monitoring, manufacturers cannot rely directly on monitoring, statistics, or periodic aseptic-processing simulations to ensure a sterility assurance level.

Environmental monitoring is usually performed by personnel and thus requires operator intervention. As a result, environmental monitoring can both increase the risk of contamination and also give false-positive results. Thus, intensive monitoring is unwarranted, particularly in the ISO 5 environments that are used in the most critical zones of aseptic processing.

A number of sampling methods can be used to assess and control the microbiological status of controlled environments for aseptic processing. At present, nearly all of these methods rely on the growth and recovery of microorganisms, many of which can be in a damaged state caused by environmental stress and therefore may be difficult to recover. The numerical values for air, surface, and personnel monitoring included in this chapter are not intended to represent limits or specifications but are strictly informational. Because of the variety of microbiological sampling equipment and methods, it is not scientifically reasonable to suggest that the attainment of these values guarantees microbial control or that excursions beyond values in this chapter indicate a loss of control. The assessment of risks associated with manufacturing environments must be made over a significant period; and in each case, the contamination recovery rate metric should be established on the basis of a review of actual findings within the facility. The objective of each user should be to use contamination recovery rates to track ongoing performance and to refine the microbiological control program to foster improvements. When optimum operational conditions are achieved within a facility, contamination recovery rate levels typically become relatively stable within a normal range of variability.

There are no standard methods for air sampling, and available literature indicates that air-sampling methods are highly variable. It should not be assumed that similar sample volumes taken by different methods will produce similar rates of recovery. Many factors can affect microbial recovery and survival, and different air sampler suppliers may have designed their systems to meet different requirements. Also, sample-to-sample variation in microbial sampling can be extensive. Limited data are available regarding the accuracy, precision, sensitivity, and limits of detection of monitoring methods used in the aseptic processing of healthcare products.

Surface sampling methods are also not standardized. Different media are employed, and in the case of swabs, different results have been reported for wet and dry swab methods and contact plates. Replicate sample contact plates should be expected to give similar results under identical conditions, but rates of recovery have been reported to be both lower than expected and highly variable. In general, surface monitoring has been found to recover <50%, even when used with relatively high inoculum levels on standardized coupons. In actual production environments where organisms are stressed to varying degrees, recovery rates may be lower.

ADVANCED ASEPTIC TECHNOLOGIES

Advanced aseptic technologies can be defined as those that do not rely on the direct intervention of human operators during processing. At present, technologies such as isolators, blow/fill/seal, and closed RABS (designs that are never opened during setup or operation) may be considered advanced aseptic technologies, provided that direct intervention by gowned personnel is disallowed during processing. In recent years, isolator technology has found a broad acceptance in healthcare manufacturing. Isolators and closed RABS effectively separate the operator from the critical aseptic processing environment. Because these systems substantially reduce contamination risk, their microbiological control levels are higher than those of conventional clean rooms that have comparable particulate air classification level, for example, ISO 5.

CLEAN ROOM CLASSIFICATION FOR ASEPTIC PROCESSING ENVIRONMENTS

The design and construction of clean rooms and controlled environments are covered in ISO 14644 series. This standard defines the performance of a clean environment with respect to the concentration of total particulates per unit volume. ISO 14644-1 stipulates the total particulate counts allowed for a clean environment to meet the defined air quality classifications. The reader is referred to this standard regarding the design characteristics and certification of clean environments.

Pharmaceutical manufacturers are concerned with nonviable particulate contamination in injectable products (see *Particulate Matter in Injections* ⟨788⟩). Unlike microbial contamination in which experimental data suggest that humans are the only significant source, nonviable particulates can arise both from humans and from processing equipment. Studies indicate that gowned humans slough particulate and microbial contamination at a rather consistent rate. However, the relationship between microbial (viable) and nonviable contamination does not hold for particulates shed by processing equipment. Where equipment is the primary source of particulate matter, the resulting particulates are essentially all nonviable.

The argument that if fewer total particulates are present in a clean room, it is less likely that airborne microorganisms will be present is true only if human operators are the source of particulate matter. It is not possible to clearly distinguish between background total particulate contamination generated largely by mechanical operations and the total particulates contributed by personnel. Thus, it is both commonplace and proper for clean-room environmental monitoring programs to consist of both a total particulate component and a microbiological component. *Table 1* describes the clean room classifications commonly used in the pharmaceutical industry. In aseptic processing, clean environments of ISO 14644-1 Classes 5–8 are typically used.

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Table 1. Airborne Total Particulate Cleanliness Classes[a]

ISO Class[b]	Particles ≥0.5 µm/m³
ISO 5	3520
ISO 6	35,200
ISO 7	352,000
ISO 8	3,520,000

[a] Taken from ISO International Standard 14644 Part 1, published by the International Organization for Standardization, May 1999.
[b] The four ISO 14644-1 classes correspond closely to former U.S. Federal Standard 209E classifications. The relationships are ISO 5/Class 100, ISO 6/Class 1000, ISO 7/Class 10,000, and ISO 8/Class 100,000.

Isolators and closed RABS present a different picture, because personnel are excluded from the aseptic processing environment and manipulations are made using glove-and-sleeve assemblies and half-suits made of thick, flexible plastic (such as polyvinyl chloride or synthetic rubber). Personnel have far less effect on the microbial quality of the environment within an isolator enclosure than in clean room environments. Some users have chosen to operate RABS in a manner that allows open, direct human intervention. In an open operational state, these systems are more similar in operation to conventional clean rooms and therefore cannot be considered advanced aseptic processing systems. In an open RABS, the ability of operators to adversely affect microbial contamination risk is higher than with closed RABS or isolators.

Specifications for air changes per hour and air velocities are not included in ISO 14644, nor were they included in Federal Standard 209E. Typically, ISO Class 8/Class 100,000 rooms are designed to provide a minimum of 20 air changes per hour; ISO Class 7/Class 10,000 rooms are designed to provide more than 50 air changes per hour; and ISO Class 5/Class 100 clean rooms provide more than 100 air changes per hour. The design of some facility criteria may differ. By diluting and removing contaminants, large volumes of air are likely to reduce airborne contamination in aseptic production. Optimum conditions vary considerably, depending on process characteristics, particularly the amount of contamination derived from personnel. These specifications should be used only as a guide in the design and operation of clean rooms, because the precise correlations among air changes per hour, air velocity, and microbial control have not been satisfactorily established experimentally.

Manufacturers should maintain a predominantly unidirectional flow of air (either vertical or horizontal) in a staffed Class 5 clean room environment, particularly when products, product containers, and closures are exposed. In the evaluation of air movement within a clean room, studying airflow visually by smoke studies or other suitable means is probably more useful than using absolute measures of airflow velocity and change rates. Risk assessment models are another useful way of reducing contamination risk and should be considered.

Air velocity and change rates are far less important in isolators or closed RABS than in clean rooms because personnel are more carefully separated from the product, product containers, and closures. Air velocities substantially lower than those used in human-scale clean rooms have proved adequate in isolator systems and may be appropriate in RABS as well. In zones within isolators where particulate matter poses a hazard to product quality, predominantly vertical or horizontal unidirectional airflow can be maintained. Experience has shown that well-controlled mixing or turbulent airflow is satisfactory for many aseptic processes and for sterility testing within isolators (see *Sterility Testing—Validation of Isolator Systems* ⟨1208⟩).

IMPORTANCE OF A MICROBIOLOGICAL EVALUATION PROGRAM FOR CONTROLLED ENVIRONMENTS

Monitoring of total particulate count in controlled environments, even with the use of electronic instrumentation on a continuous basis, does not provide information on the microbiological content of the environment. The basic limitation of particulate counters is that they measure particles of 0.5 µm or larger. While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of 10–20 µm. Particulate counts as well as microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling. Monitoring the environment for nonviable particulates and microorganisms is an important control function because they both are important in achieving product compendial requirements for *Foreign and Particulate Matter* and *Sterility* in *Injections and Implanted Drug Products* ⟨1⟩.

Total particulate monitoring may provide a better means of evaluating the overall quality of the environment in isolators and closed RABS than in most conventional clean rooms. The superior exclusion of human-borne contamination provided by an isolator results in an increased proportion of nonviable particulates. Total particulate counting in an isolator is likely to provide an immediate indicator of changes in contamination level. Microbial monitoring programs should assess the effectiveness of cleaning and sanitization practices by and of personnel who could have an impact on the bioburden. Because isolators are typically decontaminated using an automatic vapor or gas generation system, microbial monitoring is much less important in establishing their efficiency in eliminating bioburden. These automatic decontamination systems are validated directly, using an appropriate biological indicator challenge, and are controlled to defined exposure parameters during routine use to ensure consistent decontamination.

Microbial monitoring cannot and need not identify and quantify all microbial contaminants in these controlled environments. Microbiological monitoring of a clean room is technically a semiquantitative exercise, because a truly quantitative evaluation of the environment is not possible, given the limitations in sampling equipment. Both the lack of precision of enumeration methods and the restricted sample volumes that can be effectively analyzed suggest that environmental monitoring is incapable of providing direct quantitative information about sterility assurance. Analysts should remember that no microbiological sampling plan can prove the absence of microbial contamination, even when no viable contamination is recovered. The absence of growth on a microbiological sample means only that growth was not discovered; it does not mean that the environment is free of contamination.

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Routine microbial monitoring should provide sufficient information to demonstrate that the aseptic processing environment is operating in an adequate state of control. The real value of a microbiological monitoring program lies in its ability to confirm consistent, high-quality environmental conditions at all times. Monitoring programs can detect changes in the contamination recovery rate that may be indicative of changes in the state of control within the environment.

Environmental microbial monitoring and analysis of data by qualified personnel can assist in ensuring that a suitable state of control is maintained. The environment should be sampled during normal operations to allow the collection of meaningful, process-related data. Microbial sampling should occur when materials are in the area, processing activities are ongoing, and a full complement of personnel is working within the aseptic processing environment.

Microbial monitoring of manufacturing clean rooms, RABS, and isolators should include compressed gases, surfaces, room or enclosure air, and any other materials and equipment that might produce a risk of contamination. The analysis of contamination trends in an aseptic environment has long been a component of the environmental control program. In aseptic processing environments and particularly in ISO Class 5 environments, contamination is infrequently observed. In isolator enclosures, contamination is rarer still because of superior exclusion of human-borne contamination. Because of the criticality of these environments, even minor changes in the contamination incident rates may be significant, and manufacturers should frequently and carefully review monitoring data. In less critical environments, microbial contamination may be higher, but changes in recovery rates should be noted, investigated, and corrected. Isolated recoveries of microorganisms should be considered a normal phenomenon in conventional clean rooms, and these incidents generally do not require specific corrective action, because it is almost certain that investigations will fail to yield a scientifically verifiable cause. Because sampling itself requires an aseptic intervention in conventional clean rooms, any single uncorrelated contamination event could be a false positive.

When contamination recovery rates increase from an established norm, process and operational investigation should take place. Investigations will differ depending on the type and processing of the product manufactured in the clean room, RABS, or isolator. Investigation should include a review of area maintenance documentation; sanitization/decontamination documentation; the occurrence of nonroutine events; the inherent physical or operational parameters, such as changes in environmental temperature and relative humidity; and the training status of personnel.

In closed RABS and isolator systems, the loss of glove integrity or the accidental introduction of material that has not been decontaminated are among the most probable causes of detectable microbial contamination. Following the investigation, actions should be taken to correct or eliminate the most probable causes of contamination. Because of the relative rarity of contamination events in modern facilities, the investigation often proves inconclusive. When corrective actions are undertaken, they may include reinforcement of personnel training to emphasize acceptable gowning and aseptic techniques and microbial control of the environment. Some additional microbiological sampling at an increased frequency may be implemented, but this may not be appropriate during aseptic processing because intrusive or overly intensive sampling may entail an increased contamination risk. When additional monitoring is desirable, it may be more appropriate during process simulation studies. Other measures that can be considered to better control microbial contamination include additional sanitization, use of different sanitizing agents, and identification of the microbial contaminant and its possible source.

In any aseptic environment, conventional or advanced, the investigation and the rationale for the course of action chosen as a result of the investigation must be carefully and comprehensively documented.

PHYSICAL EVALUATION OF CONTAMINATION CONTROL EFFECTIVENESS

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation of clean rooms vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a structured approach to physical risk assessment may be appropriate.

One such method has been developed by Ljundqvist and Reinmüller. This method, known as the L-R method, challenges the air ventilation system by evaluating both airflow and the ability of an environment to dilute and remove airborne particles. In the L-R method, a smoke generator allows analysts to visualize the air movements throughout a clean room or a controlled environment, including vortices or turbulent zones, and the airflow pattern can be fine-tuned to minimize these undesirable effects. Following visual optimization of airflow, particulate matter is generated close to the critical zone and sterile field. This evaluation is done under simulated production conditions but with equipment and personnel in place. This type of test can also be used to evaluate the ability of RABS and isolator systems, particularly around product exit ports in these systems, to resist the effects of contamination.

Visual evaluation of air movement within clean rooms is a subjective process. Complete elimination of turbulence or vortices is not possible in operationing clean rooms that contain personnel and equipment. Air visualization is simply one step in the effort to optimize clean room operations and is not a definitive pass/fail test, because acceptable or unacceptable conditions are not readily definable.

Proper testing and optimization of the physical characteristics of the clean room, RABS, or isolator are essential before implementation of the microbiological monitoring program. Assurance that the clean room, RABS, or isolator is in compliance with its predetermined engineering specifications provides confidence that the ability of the facility systems and operating practices to control the bioburden and nonviable particulate matter are appropriate for the intended use. These tests should be repeated during routine certification of the clean room or advanced aseptic processing systems, and whenever significant changes are made to the operation, such as personnel flow, equipment operation, material flow, air-handling systems, or equipment layout.

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TRAINING OF PERSONNEL

Good personnel performance plays an essential role in the control of contamination, proper training and supervision are central to contamination control. Aseptic processing is the most critical activity conducted in microbiological controlled environments, and manufacturers must pay close attention to details in all aspects of this endeavor. Rigorous discipline and strict supervision of personnel are essential in order to ensure a level of environmental quality appropriate for aseptic processing.

Training of all personnel working in controlled environments is critical. This training is equally important for personnel responsible for the microbial monitoring program, because contamination of the clean working area could inadvertently occur during microbial sampling. In highly automated operations, monitoring personnel may be the employees who have the most direct contact with the critical surfaces and zones within the processing area. Microbiological sampling has the potential to contribute to microbial contamination caused by inappropriate sampling techniques or by placing personnel in or near the critical zone. A formal training program is required to minimize this risk. This training should be documented for all personnel who enter controlled environments. Interventions should always be minimized, including those required for monitoring activities; but when interventions cannot be avoided, they must be conducted with aseptic technique that approaches perfection as closely as possible.

Management of the facility must ensure that personnel involved in operations in clean rooms and advanced aseptic processing environments are well versed in relevant microbiological principles. The training should include instruction about the basic principles of aseptic technique and should emphasize the relationship of manufacturing and handling procedures to potential sources of product contamination. Those supervising, auditing, or inspecting microbiological control and monitoring activities should be knowledgeable about the basic principles of microbiology, microbial physiology, disinfection and sanitation, media selection and preparation, taxonomy, and sterilization. The staff responsible for supervision and testing should have academic training in medical or environmental microbiology. Sampling personnel as well as individuals working in clean rooms should be knowledgeable about their responsibilities in minimizing the release of microbial contamination. Personnel involved in microbial identification require specialized training about required laboratory methods. Additional training about the management of collected data must be provided. Knowledge and understanding of applicable standard operating procedures are critical, especially those procedures relating to corrective measures taken when environmental conditions require. Understanding of contamination control principles and each individual's responsibilities with respect to good manufacturing practices (GMPs) should be an integral part of the training program, along with training in conducting investigations and in analyzing data.

The only significant sources of microbial contamination in aseptic environments are the personnel. Because operators disperse contamination and because the ultimate objective in aseptic processing is to reduce end-user risk, only healthy individuals should be permitted access to controlled environments. Individuals who are ill must not be allowed to enter an aseptic processing environment, even one that employs advanced aseptic technologies such as isolators, blow/fill/seal, or closed RABS.

The importance of good personal hygiene and a careful attention to detail in aseptic gowning cannot be overemphasized. Gowning requirements differ depending on the use of the controlled environment and the specifics of the gowning system itself. Aseptic processing environments require the use of sterilized gowns with the best available filtration properties. The fullest possible skin coverage is desirable, and sleeve covers or tape should be considered to minimize leaks at the critical glove–sleeve junction. Exposed skin should never be visible in conventional clean rooms under any conditions. The personnel and gowning considerations for RABS are essentially identical to those for conventional clean rooms.

Once employees are properly gowned, they must be careful to maintain the integrity of their gloves, masks, and other gown materials at all times. Operators who work with isolator systems are not required to wear sterilized clean-room gowns, but inadequate aseptic technique and employee-borne contamination are the principal hazards to safe aseptic operations in isolators, as well as RABS, and in conventional clean rooms. Glove-and-sleeve assemblies can develop leaks that can allow the mechanical transfer of microorganisms to the product. A second glove, worn either under or over the primary isolator/RABS glove, can provide an additional level of safety against glove leaks or can act as a hygienic measure. Also, operators must understand that aseptic technique is an absolute requirement for all manipulations performed with gloves within RABS and isolator systems.

The environmental monitoring program, by itself, cannot detect all events in aseptic processing that might compromise the microbiological quality of the environment. Therefore, periodic media-fill or process simulation studies are necessary, as is thorough ongoing supervision, to ensure that appropriate operating controls and training are effectively maintained.

CRITICAL FACTORS IN THE DESIGN AND IMPLEMENTATION OF A MICROBIOLOGICAL ENVIRONMENTAL MONITORING PROGRAM

Since the advent of comprehensive environmental monitoring programs, their applications in capturing adverse trends or drifts has been emphasized. In a modern aseptic processing environment—whether an isolator, RABS, or conventional clean room—contamination has become increasingly rare. Nevertheless, a monitoring program should be able to detect a change from the validated state of control in a facility and to provide information for implementing appropriate countermeasures. An environmental monitoring program should be tailored to specific facilities and conditions. It is also helpful to take a broad perspective in the interpretation of data. A single uncorrelated result on a given day may not be significant in the context of the technical limitations associated with aseptic sampling methods.

Selection of Growth Media

A general microbiological growth medium such as soybean–casein digest medium (SCDM) is suitable for environmental monitoring in most cases because it supports the growth of a wide range of bacteria, yeast, and molds. This medium can be supplemented with additives to overcome or to minimize the effects of sanitizing agents or of antibiotics. Manufacturers should

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consider the specific detection of yeasts and molds. If necessary, general mycological media such as Sabouraud's, modified Sabouraud's, or inhibitory mold agar can be used. In general, monitoring for strict anaerobes is not performed, because these organisms are unlikely to survive in ambient air. However, micro-aerophilic organisms may be observed in aseptic processing. Should anoxic conditions exist or if investigations warrant (e.g., identification of these organisms in sterility testing facilities or *Sterility Tests* ⟨71⟩ results), monitoring for micro-aerophiles and organisms that grow under low-oxygen conditions may be warranted. The ability of any media used in environmental monitoring, including those selected to recover specific types of organisms, must be evaluated for their ability to support growth, as indicated in ⟨71⟩.

Selection of Culture Conditions

Time and incubation temperatures are set once the appropriate media have been selected. Typically, for general microbiological growth media such as SCDM, incubation temperatures in the ranges of approximately 20°–35° have been used with an incubation time of not less than 72 hours. Longer incubation times may be considered when contaminants are known to be slow growing. The temperature ranges given above are by no means absolute. Mesophilic bacteria and mold common to the typical facility environment are generally capable of growing over a wide range of temperatures. For many mesophilic organisms, recovery is possible over a range of approximately 20°. In the absence of confirmatory evidence, microbiologists may incubate a single plate at both a low and a higher temperature. Incubating at the lower temperature first may compromise the recovery of Gram-positive cocci that are important because they are often associated with humans.

Sterilization processes for preparing growth media should be validated. When selective media are used for monitoring, incubation conditions should reflect published technical requirements. Contamination should not be introduced into a manufacturing clean room as a result of using contaminated sampling media or equipment. Of particular concern is the use of aseptically prepared sampling media. Wherever possible, sampling media and their wrappings should be terminally sterilized by moist heat, radiation, or other suitable means. If aseptically prepared media must be used, analysts must carry out preincubation and visual inspection of all sampling media before introduction into the clean room. The reader is referred to *Microbiological Best Laboratory Practices* ⟨1117⟩ for further information regarding microbiology laboratory operations and control.

ESTABLISHMENT OF SAMPLING PLAN AND SITES

During initial startup or commissioning of a clean room or other controlled environment, specific locations for air and surface sampling should be determined. Locations considered should include those in proximity of the exposed product, containers, closures, and product contact surfaces. In aseptic processing, the area in which containers, closures, and product are exposed to the environment is often called the *critical zone*—the critical zone is always ISO 5. For aseptic operations the entire critical zone should be treated as a sterile field. A nonsterile object, including the gloved hands of clean room personnel or an RABS/isolator glove, should never be brought into contact with a sterile product, container closure, filling station, or conveying equipment before or during aseptic processing operations. Operators and environmental monitoring personnel should never touch sterile parts of conveyors, filling needles, parts hoppers, or any other equipment that is in the product-delivery pathway. This means that surface monitoring on these surfaces is best done at the end of production operations.

The frequency of sampling depends on the manufacturing process conducted within an environment. Classified environments that are used only to provide a lower overall level of bioburden in nonsterile product manufacturing areas require relatively infrequent environmental monitoring. Classified environments in which closed manufacturing operations are conducted, including fermentation, sterile API processing, and chemical processes, require fewer monitoring sites and less frequent monitoring because the risk of microbial contamination from the surrounding environment is comparatively low. Microbiological monitoring of environments in which products are filled before terminal sterilization is generally less critical than the monitoring of aseptic processing areas. The amount of monitoring required in filling operations for terminal sterilization depends on the susceptibility of the product survival and the potential for proliferation of microbial contamination. The identification and estimated number of microorganisms that are resistant to the subsequent sterilization may be more critical than the microbiological monitoring of the surrounding manufacturing environments.

It is not possible to recommend microbial control levels for each type of manufacturing environment. The levels established for one ISO Class 7 environment, for example, may be inappropriate for another ISO Class 7 environment, depending on the production activities undertaken in each. The user should conduct a prospective risk analysis and develop a rationale for the sampling locations and frequencies for each controlled environment. The classification of a clean room helps establish control levels, but that does not imply that all rooms of the same classification should have the same control levels and the same frequency of monitoring. Monitoring should reflect the microbiological control requirements of manufacturing or processing activities. Formal risk assessment techniques can result in a scientifically valid contamination control program.

Table 2 suggests frequencies of sampling in decreasing order of frequency and in relation to the criticality or product risk of the area being sampled. This table distinguishes between aseptic processing where personnel are aseptically gowned and those where a lesser gowning is appropriate. Environmental monitoring sampling plans should be flexible with respect to monitoring frequencies, and sample plan locations should be adjusted on the basis of the observed rate of contamination and ongoing risk analysis. On the basis of long-term observations, manufacturers may increase or decrease sampling at a given location or eliminate a sampling location altogether. Oversampling can be as deleterious to contamination control as undersampling, and careful consideration of risk and reduction of contamination sources can guide the sampling intensity.

Table 2. Suggested Frequency of Sampling for Aseptic Processing Areas[a]

Sampling Area/Location	Frequency of Sampling
Clean Room/RABS	
Critical zone (ISO 5 or better)	

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Table 2. Suggested Frequency of Sampling for Aseptic Processing Areas[a] *(continued)*

Sampling Area/Location	Frequency of Sampling
Active air sampling	Each operational shift
Surface monitoring	At the end of the operation
Aseptic area adjacent critical zone	
All sampling	Each operating shift
Other nonadjacent aseptic areas	
All sampling	Once per day
Isolators	
Critical zone (ISO 5 or better)	
Active air sampling	Once per day
Surface monitoring	At the end of the campaign
Nonaseptic areas surrounding the isolator	
All sampling	Once per month

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

SELECTION OF SAMPLE SITES WITHIN CLEAN ROOMS AND ASEPTIC PROCESSING AREAS

ISO 14644 suggests a grid approach for the total particulate air classification of clean rooms. This approach is appropriate for certifying the total particulate air quality performance against its design objective. Grids may also have value in analyzing risk from microbial contamination, although in general, grids that have no personnel activity are likely to have low risk of contamination. Microbial contamination is strongly associated with personnel, so microbiological monitoring of unstaffed environments is of limited value.

Microbiological sampling sites are best selected with consideration of human activity during manufacturing operations. Careful observation and mapping of the clean room during the qualification phase can provide useful information concerning the movement and positioning of personnel. Such observation can also yield important information about the most frequently conducted manipulations and interventions.

The location and movement of personnel within the clean room correlate with contamination risk to the environment and to the processes conducted within that environment. Sample sites should be selected so that they evaluate the impact of personnel movement and work within the area, particularly interventions and manipulations within the critical zone.

The most likely route of contamination is airborne, so the samples most critical to risk assessment are those that relate to airborne contamination near exposed sterile materials. Other areas of concern are entry points where equipment and materials move from areas of lower classification to those of higher classification. Areas within and around doors and airlocks should be included in the monitoring scheme. It is customary to sample walls and floors, and indeed sampling at these locations can provide information about the effectiveness of the sanitization program. Sampling at these locations can take place relatively infrequently, because contamination there is unlikely to affect product. Operators should never touch floors and walls, so mechanical transmission of contamination from these surfaces to critical areas where product is exposed should not occur.

Manufacturers typically monitor surfaces within the critical zone, although this should be done only at the end of operations. Residues of media or diluent from wet swabs should be avoided on surfaces, because they could lead to microbial proliferation. Also, cleaning surfaces to remove diluent or media requires personnel intervention and movements that can result in release of microbial contamination into the critical zone and can disrupt airflow.

MICROBIOLOGICAL CONTROL PARAMETERS IN CLEAN ROOMS, ISOLATORS, AND RABS

Since the early 1980s, manufacturers have established alert and action levels for environmental monitoring. In recent years the numerical difference between alert and action levels has become quite small, especially in ISO 5 environments. Growth and recovery in microbiological assays have normal variability in the range of ±0.5 $\log_{10}$. Studies on active microbiological air samplers indicate that variability of as high as tenfold is possible among commonly used sampling devices. As a result of this inherent variability and indeterminate sampling error, the supposed differences between, for example, an alert level of 1 cfu and an action level of 3 cfu are not analytically significant. Treating differences that are within expected, and therefore, normal ranges as numerically different is not scientifically valid and can result in unwarranted activities. In a practical sense, numerical values that vary by as much as five- to tenfold may not be significantly different.

Because of the limited accuracy and precision of microbial growth and recovery assays, analysts can consider the frequency with which contamination is detected rather than absolute numbers of cfu detected in any single sample. Also, a cfu is not a direct enumeration of microorganisms present but rather is a measure of contamination that may have originated from a clump of organisms.

Mean contamination recovery rates should be determined for each clean room environment, and changes in contamination recovery rate at a given site or within a given room may indicate the need for corrective action. Within the ISO 5 critical zone, airborne and surface contamination recovery rates of 1% or less should be attainable with current methods. Contamination

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recovery rates for closed RABS and isolator systems should be significantly lower still and can be expected to be <0.1%, on the basis of published monitoring results.

Contamination observed at multiple sites in an environment within a single sampling period may indicate increased risk to product and should be carefully evaluated. The appearance of contamination nearly simultaneously at multiple sites could also arise from poor sampling technique, so careful review is in order before drawing conclusions about potential loss of control. Resampling an environment several days after contamination is of little value, because the conditions during one sampling occasion may not be accurately duplicated during another.

Surface samples may also be taken from clean room garments. Personnel sampling should be emphasized during validation and is best done at the completion of production work in order to avoid adventitious contamination of the garments. In this case the average should be <1% for these sample sites as well. Gloves on closed RABS and isolators should meet the more rigorous expectation of <0.1% contamination recovery rates.

Because of the inherent variability of microbial sampling methods, contamination recovery rates are a more useful measure of trending results than is focusing on the number of colonies recovered from a given sample. *Table 3* provides recommended contamination recovery rates for aseptic processing environments. The incident rate is the rate at which environmental samples are found to contain microbial contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number. In other words, 99% of the samples taken are completely free of contamination. Contamination recovery rates that are higher than those recommended in *Table 3* may be acceptable in rooms of similar classification that are used for lower-risk activities. Action should be required when the contamination recovery rate trends above these recommendations for a significant time.

Table 3. Suggested Initial Contamination Recovery Rates in Aseptic Environments[a]

Room Classification	Active Air Sample (%)	Settle Plate (9 cm) 4 h Exposure (%)	Contact Plate or Swab (%)	Glove or Garment (%)
Isolator/Closed RABS (ISO 5 or better)	<0.1	<0.1	<0.1	<0.1
ISO 5	<1	<1	<1	<1
ISO 6	<3	<3	<3	<3
ISO 7	<5	<5	<5	<5
ISO 8	<10	<10	<10	<10

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

Detection frequency should be based on actual monitoring data and should be retabulated monthly. Action levels should be based on empirical process capability. If detection frequencies exceed the recommendations in *Table 3* or are greater than established process capability, then corrective actions should be taken. Corrective actions may include but are not limited to the following:

- Revision of the sanitization program, including selection of antimicrobial agents, application methods, and frequencies
- Increased surveillance of personnel practices, possibly including written critiques of aseptic methods and techniques
- Review of microbiological sampling methods and techniques

When higher-than-typical recovery levels for glove and garment contamination are observed, additional training for gowning practices may be indicated.

SIGNIFICANT EXCURSIONS

Excursions beyond approximately 15 cfu recovered from a single ISO 5 sample, whether from airborne, surface, or personnel sources, should happen very infrequently. When such ISO 5 excursions do occur, they may be indicative of a significant loss of control when they occur within the ISO 5 critical zone in close proximity to product and components. Thus, any ISO 5 excursion >15 cfu should prompt a careful and thorough investigation.

A key consideration for an abnormally high number of recovered colonies is whether this incident is isolated or can be correlated with other recoveries. Microbiologists should review recovery rates for at least two weeks before the incident of abnormally high recovery so that they can be aware of other recoveries that might indicate an unusual pattern. Microbiologists should carefully consider all recoveries, including those that are in the more typical range of 1–5 cfu. The identity of the organisms recovered is an important factor in the conduct of this investigation.

In the case of an isolated single excursion, establishing a definitive cause probably will not be possible, and only general corrective measures can be considered. It is never wise to suggest a root cause for which there is no solid scientific evidence. Also, there should be an awareness of the variability of microbial analysis. Realistically, there is no scientific reason to treat a recovery of 25 cfu as statistically different from a recovery of 15 cfu. A value of 15 cfu should not be considered significant in terms of process control, because realistically there is no difference between a recovery of 14 cfu and one of 15 cfu. Microbiologists should use practical scientific judgment in their approach to excursions.

FURTHER CONSIDERATIONS ABOUT DATA INTERPRETATION

In the high-quality environments required for aseptic processing, detection frequency typically is low. As can be seen from the rates recommended in *Table 3*, the majority of samples taken in an aseptic processing area will yield a recovery of zero

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contamination. In the most critical areas within an aseptic processing operation, it is expected that less than 1% of the samples will yield any recoverable contamination. In the most advanced of modern aseptic operations that use separative technologies such as isolators or closed RABS, the recovery rate will approach zero at all times.

The microbiologist responsible for environmental control or sterility assurance should not take this to mean that the environmental quality approaches sterility. The sensitivity of any microbial sampling system in absolute terms is not known. In environmental monitoring, a result of zero means only that the result is below the limit of detection of the analytical system. A false sense of security should not be derived from the infrequency of contamination recovery in aseptic processing.

Sterility assurance is best accomplished by a focus on human-borne contamination and the facility design features that best mitigate risk from this contamination. Greatest risk mitigation can be attained by reducing or eliminating human interventions through proper equipment design and by providing sufficient air exchanges per hour for the intended personnel population of the facility. Other risk mitigation factors include effective personnel and material movement and the proper control of temperature and humidity. Secondary factors for risk mitigation include cleaning and sanitization. Risk analysis models that analyze processes prospectively to reduce human-borne contamination risk by minimizing operator interventions are more powerful tools for sterility assurance than monitoring. Environmental monitoring cannot prove or disprove in absolute terms the sterility of a lot of product. Environmental monitoring can only assure those responsible for a process that a production system is in a consistent, validated state of control. Care should be taken to avoid drawing inappropriate conclusions from monitoring results.

SAMPLING AIRBORNE MICROORGANISMS

Among the most commonly used tools for monitoring aseptic environments are impaction and centrifugal samplers. A number of commercially available samplers are listed for informational purposes. The selection, appropriateness, and adequacy of using any particular sampler are the responsibility of the user.

Slit-to-Agar Air Sampler (STA)

The unit is powered by an attached source of controllable vacuum. The air intake is obtained through a standardized slit below which is placed a slowly revolving Petri dish that contains a nutrient agar. Airborne particles that have sufficient mass impact the agar surface, and viable organisms are allowed to grow. A remote air intake is often used to minimize disturbance of unidirectional airflow.

Sieve Impactor

This apparatus consists of a container designed to accommodate a Petri dish that contains a nutrient agar. The cover of the unit is perforated with openings of a predetermined size. A vacuum pump draws a known volume of air through the cover, and airborne particles that contain microorganisms impact the agar medium in the Petri dish. Some samplers feature a cascaded series of sieves that contain perforations of decreasing size. These units allow determination of the size range distribution of particulates that contain viable microorganisms based on the size of the perforations through which the particles landed on the agar plates.

Centrifugal Sampler

The unit consists of a propeller or turbine that pulls a known volume of air into the unit and then propels the air outward to impact on a tangentially placed nutrient agar strip set on a flexible plastic base.

Sterilizable Microbiological Atrium

The unit is a variant of the single-stage sieve impactor. The unit's cover contains uniformly spaced orifices approximately 0.25 inch in size. The base of the unit accommodates one Petri dish containing a nutrient agar. A vacuum pump controls the movement of air through the unit, and a multiple-unit control center as well as a remote sampling probe are available.

Surface Air System Sampler

This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit's perforated cover over the agar contact plate and beyond the motor, where it is exhausted. Multiple mounted assemblies are also available.

Gelatin Filter Sampler

The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.

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Settling Plates

This method is still widely used as a simple and inexpensive way to qualitatively assess the environments over prolonged exposure times. Published data indicate that settling plates, when exposed for 4- to 5-hour periods, can provide a limit of detection for a suitable evaluation of the aseptic environment. Settling plates may be particularly useful in critical areas where active sampling could be intrusive and a hazard to the aseptic operation.

One of the major drawbacks of mechanical air samplers is the limited sample size of air being tested. When the microbial level in the air of a controlled environment is expected to contain extremely low levels of contamination per unit volume, at least 1 cubic meter of air should be tested in order to maximize sensitivity. Typically, slit-to-agar devices have an 80-L/min sampling capacity (the capacity of the surface air system is somewhat higher). If 1 cubic meter of air were tested, then it would require an exposure time of 15 min. It may be necessary to use sampling times in excess of 15 min to obtain a representative environmental sample. Although some samplers are reported to have high sampling volumes, consideration should be given to the potential for disruption of the airflow patterns in any critical area and to the creation of turbulence.

Technicians may wish to use remote sampling systems in order to minimize potential risks resulting from intervention by environmental samplers in critical zones. Regardless of the type of sampler used, analysts must determine that the extra tubing needed for a remote probe does not reduce the method's sensitivity to such an extent that detection of low levels of contamination becomes unlikely or even impossible.

SURFACE SAMPLING

Another component of the microbial-control program in controlled environments is surface sampling of equipment, facilities, and personnel. The standardization of surface sampling methods and procedures has not been as widely addressed in the pharmaceutical industry as has the standardization of air-sampling procedures. Surface sampling can be accomplished by the use of contact plates or by the swabbing method.

Contact plates filled with nutrient agar are used for sampling regular or flat surfaces and are directly incubated for the appropriate time and temperature for recovery of viable organisms. Specialized agar can be used for the recovery of organisms that have specific growth requirements. Microbial estimates are reported per contact plate.

The swabbing method can be used to supplement contact plates for sampling of irregular surfaces, especially irregular surfaces of equipment. The area that will be swabbed is defined with a sterile template of appropriate size. In general, it is in the range of 24–30 cm^2. After sample collection the swab is placed in an appropriate diluent or transport medium and is plated onto the desired nutrient agar. The microbial estimates are reported per swab of defined sampling area.

Surface monitoring is used as an environmental assessment tool in all types of classified environments. In ISO 5 environments for aseptic processing, surface monitoring is generally performed beside critical areas and surfaces. Component hoppers and feed chutes that contact sterile surfaces on closures and filling needles can be tested for microbial contamination. Often in conventional staffed clean rooms, these product contact surfaces are steam sterilized and aseptically assembled. The ability of operators to perform these aseptic manipulations are evaluated during process stimulations or media fills, although true validation of operator technique in this manner is not possible. Surface monitoring on surfaces that directly contact sterile parts or product should be done only after production operations are completed. Surface sampling is not a sterility test and should not be a criterion for the release or rejection of product. Because these samples must be taken aseptically by personnel, it is difficult to establish with certainty that any contamination recovered is product related.

CULTURE MEDIA AND DILUENTS

The type of medium, liquid or solid, used for sampling or plating microorganisms depends on the procedure and equipment used. Any medium used should be evaluated for suitability for the intended purpose. The most commonly used all-purpose solid microbiological growth medium is soybean–casein digest agar. As previously noted, this medium can be supplemented with chemicals that counteract the effect of various antimicrobials.

IDENTIFICATION OF MICROBIAL ISOLATES

A successful environmental control program includes an appropriate level of identification of the flora obtained by sampling. A knowledge of the flora in controlled environments aids in determining the usual microbial flora anticipated for the facility and in evaluating the effectiveness of the cleaning and sanitization procedures, methods, agents, and recovery methods. The information gathered by an identification program can be useful in the investigation of the source of contamination, especially when recommended detection frequencies are exceeded.

Identification of isolates from critical and immediately adjacent areas should take precedence over identification of microorganisms from noncritical areas. Identification methods should be verified, and ready-to-use kits should be qualified for their intended purpose.

CONCLUSION

Environmental monitoring is one of several key elements required in order to ensure that an aseptic processing area is maintained in an adequate level of control. Monitoring is a qualitative exercise, and even in the most critical applications such as aseptic processing, conclusions regarding lot acceptability should not be made on the basis of environmental sampling results alone. Environments that are essentially free of human operators generally have low initial contamination rates and maintain

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low levels of microbial contamination. Human-scale clean rooms present a very different picture. Studies conclusively show that operators, even when carefully and correctly gowned, continuously slough microorganisms into the environment. Therefore, it is unreasonable to assume that samples producing no colonies, even in the critical zone or on critical surfaces, will always be observed. Periodic excursions are a fact of life in human-scale clean rooms, but the contamination recovery rate, particularly in ISO 5 environments used for aseptic processing, should be consistently low.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous improvement of personnel operations and environmental control. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from microbial contamination.

GLOSSARY

Airborne Particulate Count (also referred to as *Total Particulate Count*): The total number of particles of a given size per unit volume of air.

Airborne Viable Particulate Count (also referred to as *Total Airborne Aerobic Microbial Count*): The recovered number of colony-forming units (cfu) per unit volume of air.

Air Changes: The frequency per unit of time (minutes, hours, etc.) that the air within a controlled environment is replaced. The air can be recirculated partially or totally replaced.

Air Sampler: Devices or equipment used to sample a measured amount of air in a specified time to quantitate the particulate or microbiological status of air in the controlled environment.

Aseptic: Technically, the absence of microorganisms, but in aseptic processing this refers to methods and operations that minimize microbial contamination in environments where sterilized product and components are filled and/or assembled.

Aseptic Processing: An operation in which the product is assembled or filled into its primary package in an ISO 5 or better environment and under conditions that minimize the risk of microbial contamination. The ultimate goal is to produce products that are as free as possible of microbial contamination.

Barrier System: Physical barriers installed within an aseptic processing room to provide partial separation between aseptically gowned personnel and critical areas subject to considerable contamination risk. Personnel access to the critical zone is largely unrestricted. It is subject to a high level disinfection.

Bioburden: Total number and identity of the predominant microorganisms detected in or on an article.

Clean Room: A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness Class. In addition, the concentration of microorganisms in the environment is monitored; each cleanliness Class defined is also assigned a microbial level for air, surface, and personnel gear.

Commissioning of a Controlled Environment: Certification by engineering and quality control that the environment has been built according to the specifications of the desired cleanliness Class and that, under conditions likely to be encountered under normal operating conditions (or worst-case conditions), it is capable of delivering an aseptic process. Commissioning includes media-fill runs and results of the environmental monitoring program.

Contamination Recovery Rate: The contamination recovery rate is the rate at which environmental samples are found to contain any level of contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number.

Controlled Environment: Any area in an aseptic process system for which airborne particulate and microorganism levels are controlled to specific levels, appropriate to the activities conducted within that environment.

Corrective Action: Actions to be performed that are according to standard operating procedures and that are triggered when certain conditions are exceeded.

Critical Zone: Typically the entire area where product and the containers and closures are exposed in aseptic processing.

Detection Frequency: The frequency with which contamination is observed in an environment. Typically expressed as a percentage of samples in which contamination is observed per unit of time.

Environmental Isolates: Microorganisms that have been isolated from the environmental monitoring program.

Environmental Monitoring Program: Documented program implemented via standard operating procedures that describes in detail the methods and acceptance criteria for monitoring particulates and microorganisms in controlled environments (air, surface, personnel gear). The program includes sampling sites, frequency of sampling, and investigative and corrective actions.

Equipment Layout: Graphical representation of an aseptic processing system that denotes the relationship between and among equipment and personnel. This layout is used in the *Risk Assessment Analysis* to determine sampling site and frequency of sampling based on potential for microbiological contamination of the product/container/closure system. Changes must be assessed by responsible managers, since unauthorized changes in the layout for equipment or personnel stations could result in increase in the potential for contamination of the product/container/closure system.

Isolator for Aseptic Processing: An aseptic isolator is an enclosure that is over-pressurized with HEPA filtered air and is decontaminated using an automated system. When operated as a closed system, it uses only decontaminated interfaces or rapid transfer ports (RTPs) for materials transfer. After decontamination they can be operated in an open manner with the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. It can be used for aseptic processing activities or for asepsis and containment simultaneously.

Material Flow: The flow of material and personnel entering controlled environments should follow a specified and documented pathway that has been chosen to reduce or minimize the potential for microbial contamination of the product/closure/container systems. Deviation from the prescribed flow could result in increase in the potential for microbial contamination. Material/personnel flow can be changed, but the consequences of the changes from a microbiological point of view should be assessed by responsible managers and must be authorized and documented.

Media Fill: Microbiological simulation of an aseptic process by the use of growth media processed in a manner similar to the processing of the product and with the same container/closure system being used.

Media Growth Promotion: Procedure that references *Growth Promotion Test of Aerobes, Anerobes, and Fungi* in *Sterility Tests* ⟨71⟩ to demonstrate that media used in the microbiological environmental monitoring program, or in *media-fill* runs, are

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capable of supporting growth of indicator microorganisms and of environmental isolates from samples obtained through the monitoring program or their corresponding ATCC strains.

Product Contact Areas: Areas and surfaces in a controlled environment that are in direct contact with either products, containers, or closures and the microbiological status of which can result in potential microbial contamination of the product/container/closure system.

Restricted Access Barrier System (RABS): An enclosure that relies on HEPA filtered air over-spill to maintain separation between aseptically gowned personnel and the operating environment. It is subject to a high level of disinfection prior to use in aseptic process. It uses decontaminated (where necessary) interfaces or RTPs for materials transfer. It allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. If opened subsequent to decontamination, its performance capability is adversely impacted.

Risk Assessment Analysis: Analysis of the identification of contamination potentials in controlled environments that establish priorities in terms of severity and frequency and that will develop methods and procedures that will eliminate, reduce, minimize, or mitigate their potential for microbial contamination of the product/container/closure system.

Sampling Plan: A documented plan that describes the procedures and methods for sampling a controlled environment; identifies the sampling sites, the sampling frequency, and number of samples; and describes the method of analysis and how to interpret the results.

Sampling Sites: Documented geographical location, within a controlled environment, where sampling for microbiological evaluation is taken. In general, sampling sites are selected because of their potential for product/container–closure contacts.

Standard Operating Procedures: Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place in a controlled environment and auxiliary environments. Deviations from standard operating procedures should be noted and approved by responsible managers.

Sterile or Aseptic Field: In aseptic processing or in other controlled environments, it is the space at the level of or above open product containers, closures, or product itself, where the potential for microbial contamination is highest.

Sterility: Within the strictest definition of sterility, an article is deemed sterile when there is complete absence of viable microorganisms. *Viable*, for organisms, is defined as having the capacity to reproduce. Absolute sterility cannot be practically demonstrated because it is technically unfeasible to prove a negative absolute. Also, absolute sterility cannot be practically demonstrated without testing every article in a batch. Sterility is defined in probabilistic terms, where the likelihood of a contaminated article is acceptably remote.

Swabs for Microbiological Sampling: Devices used to remove microorganisms from irregular or regular surfaces for cultivation to identify the microbial population of the surface. A swab is generally composed of a stick with an absorbent tip that is moistened before sampling and is rubbed across a specified area of the sample surface. The swab is then rinsed in a sterile solution to suspend the microorganisms, and the solution is transferred to growth medium for cultivation of the microbial population.

Trend Analysis: Data from a routine microbial environmental monitoring program that can be related to time, shift, facility, etc. This information is periodically evaluated to establish the status or pattern of that program to ascertain whether it is under adequate control. A trend analysis is used to facilitate decision-making for requalification of a controlled environment or for maintenance and sanitization schedules.

APPENDIX

Additional Resources

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⟨1117⟩ MICROBIOLOGICAL BEST LABORATORY PRACTICES

INTRODUCTION

Good laboratory practices in a microbiology laboratory consist of activities that depend on several principles: aseptic technique, control of media, control of test strains, operation and control of equipment, diligent recording and evaluation of data, and training of the laboratory staff. Because of the inherent risk of variability in microbiology data, reliability and reproducibility are dependent on the use of accepted methods and adherence to good laboratory practices.

MEDIA PREPARATION AND QUALITY CONTROL

Media Preparation

Culture media are the basis for most microbiological tests. Safeguarding the quality of the media is therefore critical to the success of the microbiology laboratory. Media preparation, proper storage, and quality control testing can ensure a consistent supply of high-quality media.

It is important to choose the correct media or components in making media based on the use of accepted sources or references for formulas. The manufacturer's formula and instructions for preparation routinely accompany dehydrated media and ready-made media. Because different media types may have different preparation requirements (e.g., heating, additives, and pH adjustment), it is important to follow these instructions to ensure preparation of acceptable media quality. A certificate of analysis describing expiration dating and recommended storage conditions accompanies ready-made media, as well as the quality control organisms used in growth-promotion and selectivity testing of that media.

Water is the universal diluent for microbiological media. Purified Water is most often used for media preparation, but in certain cases the use of deionized or distilled water may be appropriate. Water of lesser quality should not be used for microbiological media preparation. The volume of the water used should be recorded.

Consistent preparation of media requires accurate weighing of dehydrated media or media constituents. A calibrated balance with the appropriate weight range for the ingredients should be used (See *Weighing on an Analytical Balance* ⟨1251⟩). Clean weighing containers and tools (such as spatulas) should be used to prevent foreign substances from entering the formulation. The weight of the components should be recorded.

Dehydrated media should be thoroughly dissolved in water before dispensing and sterilization. If heating is necessary to help dissolve the media, care should be taken not to overheat media, because all culture media, to a greater or lesser extent, are heat-sensitive. Equipment used in the preparation of media should be appropriate to allow for controlled heating, constant agitation, and mixing of the media. Darkening of media (Maillard-type reaction or nonenzymatic browning) is a general indication of overheating. When adding required supplements to media, adequate mixing of the medium after adding the supplement should be performed.

Preparation of media in poorly cleaned glassware can allow inhibitory substances to enter the media. Inhibitory substances can come from detergent residue after cleaning glassware or from prior materials used in the glassware. Be sure that the cleaning process removes debris and foreign matter, and that the detergent is thoroughly rinsed out with Purified Water. See *Cleaning Glass Apparatus* ⟨1051⟩ for additional guidance.

Sterilization of media should be performed within the parameters provided by the manufacturer or validated by the user. Commercially prepared media should provide documentation of the sterilization method used. Autoclaving by moist heat is the preferred sterilization technique, except in instances when boiling is required in order to avoid deterioration of heat-labile components of the media. Sterilization by filtration may also be appropriate for some formulations.

The effects of the sterilization method and conditions on the media should be validated by sterility and growth-promotion testing of the media. In addition, if sterilized by moist heat, the autoclave cycle should be validated to ensure proper heat distribution for selected loads and volumes. Typically, manufacturers recommend using an autoclave cycle of 121° for 15 minutes using a validated autoclave. These conditions apply to time at temperature of the media. As container size and the load configuration of the autoclave will influence the rate of heating, longer cycles may be required for larger loads. However, the sterilization time will be dependent on the media volume and autoclave load. Sterilization cycles in which the autoclave is slow to come up to temperature may result in overheating of the media. Therefore, care must be taken to validate a sterilization cycle, balancing the need for sterile media against the tendency of the media to degrade under excessive heating. Storage of the media in the autoclave after the liquid cycle is completed is not recommended after cooling, as it may damage the media. Improper heating or sterilizing conditions—for commercially prepared or internally prepared media—may result in a difference in color change, loss of clarity, altered gel strength, or pH drift from the manufacturer's recommended range, as well as reduced growth-promotion activity and/or selectivity.

The pH of each batch of medium should be confirmed after it has cooled to room temperature (20°–25°) by aseptically withdrawing a sample for testing. Refrigerated purchased media should be allowed to warm up to ambient room temperature if it is to be checked for pH confirmation. A flat pH probe is recommended for agar surfaces, and an immersion probe is

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recommended for liquids. See *pH* ⟨791⟩ for guidance with pH measurement and instrument calibration. The pH of media should be in a range of ±0.2 of the value indicated by the manufacturer, unless a wider range is acceptable by the validated method.

Prepared media should be checked by appropriate inspection of plates and tubes for the following:

- Cracked containers or lids
- Unequal filling of containers
- Dehydration resulting in cracks or dimpled surfaces on solid medium
- Hemolysis
- Excessive darkening or color change
- Crystal formation from possible freezing
- Excessive number of bubbles
- Microbial contamination
- Status of redox indicators (if appropriate)
- Lot number and expiration date checked and recorded
- Sterility of the media
- Cleanliness of plates (lid should not stick to dish)

Media Storage

It is prudent to consider how the manufacturer or supplier transports and stores media before distribution to the end user. Manufacturers of media should use transport and storage conditions that minimize the loss of moisture, control the temperature, prevent microbial contamination, and provide mechanical protection to the prepared media.

Media should be labeled properly with batch or lot numbers, preparation and expiration dates, and media identification. Media should be stored according to the manufacturer's instructions. Media prepared in house should be stored under validated conditions. Do not store agar at or below 0°, as freezing could damage the gel structure. Protect stored media from exposure to light and excessive temperature. Before prolonged storage, agar plates should be placed into a sealed package or container to retard moisture loss.

Remelting of an original container of solid media should be performed only once to avoid media whose quality is compromised by overheating or potential contamination. It is recommended that remelting be performed in a heated water bath or by using free-flowing steam. The use of microwave ovens and heating plates is common, but care should be taken to avoid damaging media by overheating and to avoid the potential injury to laboratory personnel from glass breakage and burns. The molten agar medium should be held in a monitored water bath at a temperature of 45° to 50° for not more than 8 hours. Caution should be taken when pouring the media from a container immersed in a water bath to prevent water from the bath commingling with the poured sterile media. Wiping the exterior of the container dry before pouring may be advisable.

Disposal of used cultured media (as well as expired media) should follow local biological hazard safety procedures.

Quality Control Testing

Although growth media can be prepared in a laboratory from individual components, many laboratories, for ease of use, use dehydrated media or purchase commercially prepared media in plastic plates or glass containers. Manufacturers of media attempt to standardize raw materials from biological sources, but must constantly deal with unavoidable differences in raw materials obtained from natural sources, and therefore, lot-to-lot variability of media must be considered. In addition, the performance of media prepared in a laboratory or by a manufacturer is highly dependent on preparation and storage conditions. Improper media preparation can cause unsatisfactory conditions for microbial growth or recovery and unreliable results.

Therefore, quality control tests should be performed on all prepared media, including media associated with swabs or media in strips and other nontraditional formats. Tests routinely performed on in-house prepared media should include pH, growth promotion, inhibition, and indicative properties (as appropriate), and periodic stability checks to confirm the expiration dating.

When in-house prepared microbiological media are properly prepared and sterilized using a validated method, the growth-promotion testing may be limited to each incoming lot of dehydrated media, unless otherwise instructed by the relevant compendial method. If the media preparation procedure was not validated, then every batch of media should be subjected to growth-promotion testing. Test organisms may be selected from the appropriate compendial test chapter. In addition, microorganisms used in growth-promotion testing may be based on the manufacturer's recommendation for a particular medium, or may include representative environmental isolates (but these latter are not to be construed as compendial requirements).

Expiration dates on media should have supporting growth-promotion testing to indicate that the performance of the media still meets acceptance criteria up to and including the expiration date. The length of shelf life of a batch of media will depend on the stability of the ingredients and formulation under specified conditions, as well as the type of container and closure.

When a batch of media does not meet the requirements of growth-promotion testing, an investigation should be initiated to identify the cause. This investigation should include a corrective action plan to prevent the recurrence of the problem. Any batch of media that fails growth-promotion testing is unsuitable for use. [NOTE—Failed growth-promotion test results may not be used to negate positive test results.]

Some reagents are used for diagnostic purposes to help support identification of microbial organisms, e.g., Gram stain and oxidase test reagents. These may have attributes that can be quality control tested similar to microbiological media. Select the correct quality control standard microorganisms, following the manufacturer's instructions, and perform the testing before unknown sample diagnostic testing. All relevant diagnostic reagents should be subjected to incoming quality confirmation before use.

Special care should be taken with media that is used in sterility tests (see *Sterility Tests* ⟨71⟩ for requirements) and in environmental monitoring studies. Media used for environmental monitoring of critical areas should preferably be

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double-wrapped and terminally sterilized. If terminal sterilization is not performed, media should be subjected to 100% pre-incubation and inspection before use within a critical area. [NOTE—Growth-promotion testing for this media must be performed after the pre-incubation stage.] This will prevent extraneous contamination from being carried into controlled environments and will prevent false-positive results. A raised agar level for surface contact plates should be verified.

MAINTENANCE OF MICROBIOLOGICAL CULTURES

Biological specimens can be the most delicate standards to handle because their viability and characteristics are dependent on adequate handling and storage. Standardizing the handling and storage of cultures by the user laboratory should be done in a way that will minimize the opportunity for contamination or alteration of growth characteristics. The careful and consistent treatment of stock cultures is critically important to the consistency of microbiological test results. Cultures for use in compendial tests should be acquired from a national culture collection or a qualified secondary supplier. They can be acquired frozen, freeze-dried, on slants, or in ready-to-use forms. Confirmation of the purity of the culture and the identity of the culture should be performed before its use in quality control testing. Ready-to-use cultures should be subjected to incoming testing for purity and identity before use. The confirmation of identity for commonly used laboratory strains should ideally be done at the level of genus and species.

Preparation and resuscitation of cultures should follow the instructions of the supplier or a validated, established method. The "Seed-Lot" technique is recommended for storage of stock cultures.

The original sample from the national culture collection or a qualified secondary supplier is resuscitated and grown in an appropriate medium. Aliquots of this stock culture (the first transfer or passage) are suspended in a cryoprotective medium, transferred to vials, and frozen at –30° or below, until use. If stored at –70°, or in lyophilized form, strains may be kept indefinitely. These frozen stocks can then be used to inoculate monthly or weekly working cultures. Once opened, do not refreeze unused cell suspensions after culturing a working suspension. The unused portion should be discarded to minimize the risk of loss of viability and contamination of the stock.

The number of transfers of working control cultures should be tracked to prevent excessive subculturing that increases the risk of phenotypic alteration or mutation. The number of transfers allowable for specific compendial tests may be specified in that test. One passage is defined as the transfer of organisms from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a transfer/passage.

LABORATORY EQUIPMENT

Most equipment (incubators, water baths, and autoclaves) is subject to standard validation practices of incoming qualification, operational qualification, and performance qualification. Additionally, periodic calibration (generally annually) is commonly required. New equipment, critical to the operation of the laboratory, should be qualified according to a protocol approved by the quality assurance unit (QAU). In addition, regular cleaning and sanitization of equipment such as incubators, refrigerators, and water baths should be performed to minimize the potential for contamination in the laboratory. Door seals of incubators and refrigerators should be cleaned and checked for state of repair.

Instruments (pH meters and spectrophotometers) used in a microbiology laboratory should be calibrated on a regular schedule and tested to verify performance on a routine basis. The frequency of calibration and performance verification will vary based on the type of instrument and the importance of that equipment to the generation of data in the laboratory.

Equipment that is difficult to sanitize (such as refrigerators and incubators) should be dedicated to aseptic operations (such as storage of media for testing and incubation of sterility test samples) and live culture operations to minimize the potential for inadvertent contamination of the tests.

Autoclaves are central to the operation of the laboratory and must have proper validation in place to demonstrate adequate sterilization for a variety of operations. Autoclave resources must be available (and validated) to sterilize waste media (if performed in that laboratory) as well as the media prepared in that laboratory. The choice of one or several autoclaves is not driven by a need to separate aseptic and live operations (everything in the properly maintained autoclave is sterile after the cycle) but rather driven by resource considerations (see below).

LABORATORY LAYOUT AND OPERATIONS

Laboratory layout and design should carefully consider the requirements of good microbiological practices and laboratory safety. It is essential that cross-contamination of microbial cultures be minimized to the greatest extent possible, and it is also important that microbiological samples be handled in an environment that makes contamination highly unlikely.

In general, a laboratory should be divided into clean or aseptic areas and live culture areas. Areas in which environmental or sterile product samples are handled and incubated should be maintained completely free of live cultures, if possible. If complete separation of live and clean culture zones cannot be accomplished, then other barriers and aseptic practices should be employed to reduce the likelihood of accidental contamination. These barriers include protective clothing, sanitization and disinfection procedures, and biological safety cabinets designated for clean or aseptic operations only. Procedures for handling spills or mishaps with live cultures should be in place, and all relevant technical personnel should be trained regarding these methods.

Some samples will demonstrate microbial growth and require further laboratory analysis to identify the contaminants. When growth is detected, the sample should be taken from the clean section of the laboratory to the live culture section without undue delay. Subculturing, staining, microbial identification, or other investigational operations should be undertaken in the live culture section of the laboratory. If possible, any sample found to contain growing colonies should not be opened in the clean zone of the laboratory. Careful segregation of contaminated samples and materials will reduce false-positive results.

Staff engaged in sampling activities should not enter or work in the live culture handling section of a laboratory unless special precautions are taken, including wearing protective clothing and gloves and careful sanitizing of hands upon exiting. Ideally,

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staff assigned to sampling activities, particularly those in support of aseptic processing, should not work in the vicinity of live culture laboratory operations.

It is important to consider that microbial contamination of samples, which leads to false-positive results, is always possible unless careful aseptic precautions are taken. Facilities should be designed so that raw material and excipient sampling can be done under controlled conditions, including proper gowning and sterilized sampling equipment. It may not always be possible to sample utility systems, such as water systems, under full aseptic conditions; however, it should be noted that when samples are not taken aseptically, their reliability is inevitably compromised.

Environmental sampling methods should require minimal aseptic handling in loading and unloading sampling instruments. Whenever possible, sampling equipment should be loaded with its microbiological recovery media in the environment that is to be sampled.

All testing in laboratories used for critical testing procedures, such as sterility testing of final dosage forms, bulk product, seed cultures for biological production, or cell cultures used in biological production, should be performed under controlled conditions. Isolator technology is also appropriate for critical, sterile microbiological testing. Isolators have been shown to have lower levels of environmental contamination than manned clean rooms, and therefore, are generally less likely to produce false-positive results. Proper validation of isolators is critical both to ensure environmental integrity and to prevent the possibility of false-negative results as a result of chemical disinfection of materials brought into or used within isolators (see *Sterility Testing —Validation of Isolator Systems* ⟨1208⟩).

SAMPLE HANDLING

Viable microorganisms in most microbiology samples, particularly water, environmental monitoring and bioburden samples, are sensitive to handling and storage conditions. Critical parameters in these conditions include product (or sample) composition, container composition, time of storage, and temperature of storage. Therefore, it is important to minimize the amount of time between the sampling event and the initiation of testing and to control, as much as possible, the conditions of storage. If the sample is to be transported to a distant location for testing, then the conditions of transport (time, temperature, etc.) should be qualified as suitable for that test and sample. Guidance for water testing in this regard can be found in *Water for Pharmaceutical Purposes* ⟨1231⟩. Product mixing before sampling may need to be evaluated and applied in order to ensure microbial dispersement and representation in the sample aliquot.

All microbiological samples should be taken using aseptic techniques, including those taken in support of nonsterile products. If possible, all microbiological samples should be taken under full aseptic conditions in specialized sampling areas. The areas should be as close to the point of use as possible to minimize contamination during transit.

Samples submitted to the microbiology laboratory should be accompanied by documentation detailing source of the sample, date the sample was taken, date of sample submission, person or department responsible for the submission, and any potentially hazardous materials associated with the sample. The testing department should acknowledge receipt of the sample and reconcile the identity and number of samples as part of this sample documentation.

MICROBIOLOGICAL MEDIA INCUBATION TIMES

Incubation times for microbiological tests of less than 3 days' duration should be expressed in hours: e.g., "Incubate at 30° to 35° for 18 to 72 hours". Tests longer than 72 hours' duration should be expressed in days: e.g., "Incubate at 30° to 35° for 3 to 5 days". For incubation times expressed in hours, incubate for the minimum specified time, and exercise good microbiological judgment when exceeding the incubation time. For incubation times expressed in days, incubations started in the morning or afternoon should generally be concluded at that same time of day.

TRAINING OF PERSONNEL

Each person engaged in each phase of pharmaceutical manufacture should have the education, training, and experience to do his or her job. The demands of microbiological testing require that the core educational background of the staff, supervisors, and managers be in microbiology or a closely related biological science. They should be assigned responsibilities in keeping with their level of skill and experience.

A coherent system of standard operating procedures (SOPs) is necessary to run the microbiology laboratory. These procedures serve two purposes in a training program. Firstly, these SOPs describe the methodology that the microbiologist will follow to obtain accurate and reproducible results, and so serve as the basis for training. Secondly, by tracking the procedures in which a particular microbiologist has demonstrated proficiency, the procedure number or title also serves to identify what training the microbiologist has received specific to his or her job function.

Training curricula should be established for each laboratory staff member specific to his or her job function. He or she should not independently conduct a microbial test until qualified to run the test. Training records should be current, documenting the microbiologist's training in the current revision to the particular SOP.

Periodic performance assessment is a wise investment in data quality. This performance testing should provide evidence of competency in core activities of the microbiology laboratory such as hygiene, plating, aseptic technique, documentation, and others as suggested by the microbiologist's job function.

Microbiologists with supervisory or managerial responsibilities should have appropriate education and in-house training in supervisory skills, laboratory safety, scheduling, budgeting, investigational skills, technical report writing, relevant SOPs, and other critical aspects of the company's processes as suggested in their role of directing a laboratory function.

Competency may be demonstrated by specific course work, relevant experience, and routinely engaging in relevant continuing education. Achieving certification through an accredited body is also a desirable credential. Further, it is expected that laboratory supervisors and managers have a demonstrated level of competence in microbiology at least as high as those

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they supervise. Expertise in microbiology can be achieved by a variety of routes in addition to academic course work and accreditation. Each company is expected to evaluate the credentials of those responsible for designing, implementing, and operating the microbiology program. Companies can thus ensure that those responsible for the program understand the basic principles of microbiology, can interpret guidelines and regulations based on good science, and have access to individuals with theoretical and practical knowledge in microbiology to provide assistance in areas in which the persons responsible for the program may not have adequate knowledge and understanding. It should be noted that microbiology is a scientifically based discipline that deals with biological principles substantially different from those of analytical chemistry and engineering disciplines. Many times it is difficult for individuals without specific microbiological training to make the transition.

LABORATORY RESOURCES

The laboratory management is responsible for ensuring that the laboratory has sufficient resources to meet the existing testing requirements. This requires some proficiency in budget management and in determining appropriate measures of laboratory performance. A measure of laboratory performance is the number of investigations performed on tests conducted by the laboratory, but this measure alone is not sufficient. In addition to tracking investigations, the period of time between sample submission and initiation of testing should be tracked, as well as the period of time between end of test and report release (or test closure). Significant delays in these measures are also indications of an under-resourced laboratory staff.

The laboratory management should have sufficient budget to meet testing requirements. Particular measures of budgetary requirements will be specific to the given laboratory, but budgetary considerations related directly to the need of the laboratory for sufficient resources must be addressed to ensure reliable testing results.

DOCUMENTATION

Documentation should be sufficient to demonstrate that the testing was performed in a laboratory and by methods that were under control. This includes, but is not limited to, documentation of the following:

- Microbiologist training and verification of proficiency
- Equipment validation, calibration, and maintenance
- Equipment performance during test (e.g., 24-hour/7-day chart recorders)
- Media preparation, sterility checks, and growth-promotion and selectivity capabilities
- Media inventory and control testing
- Critical aspects of test conducted as specified by a procedure
- Data and calculations verification
- Reports reviewed by QAU or a qualified responsible manager
- Investigation of data deviations (when required)

MAINTENANCE OF LABORATORY RECORDS

Proper recording of data and studies is critical to the success of the microbiology laboratory. The over-riding principle is that the test should be performed as written in the SOP, the SOP should be written to reflect how the test is actually performed, and the laboratory notebook should provide a record of all critical details needed to reconstruct the details of the testing and confirm the integrity of the data. At a minimum, the laboratory write-up should include the following:

- Date
- Material tested
- Microbiologist's name
- Procedure number
- Document test results
- Deviations (if any)
- Documented parameters (equipment used, microbial stock cultures used, media lots used)
- Management/Second review signature

Every critical piece of equipment should be noted in the write-up, and all should be on a calibration schedule documented by SOP and maintenance records. Where appropriate, logbooks or forms should be available and supportive of the laboratory notebook records. Equipment temperatures (water baths, incubators, autoclaves) should be recorded and traceable.

The governing SOP and revision should be clearly noted in the write-up. Changes in the data should be crossed off with a single line and initialed. Original data should not be erased or covered over.

Test results should include the original plate counts, allowing a reviewer to recreate the calculations used to derive the final test results. Methods for data analysis should be detailed in cited SOPs. If charts or graphs are incorporated into laboratory notebooks, they should be secured with clear tape and should not be obstructing any data on the page. The chart or graph should be signed by the person adding the document, with the signature overlapping the chart and the notebook page. Lab notebooks should include page numbers, a table of contents for reference, and an intact timeline of use.

All laboratory records should be archived and protected against catastrophic loss. A formal record retention and retrieval program should be in place.

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INTERPRETATION OF ASSAY RESULTS

Analytical microbiological assay results can be difficult to interpret for several important reasons: (1) Microorganisms are ubiquitous in nature, and common environmental contaminants—particularly organisms associated with humans—predominate in many types of microbiological analysis; (2) the analyst has the potential to introduce contaminating organisms during sample handling or processing in the laboratory; (3) microorganisms may not be homogeneously distributed within a sample or an environment; and (4) microbiological assays are subject to considerable variability of outcome. Therefore, apparent differences from an expected outcome may not be significant.

Because of these characteristics of microbiological analysis, laboratory studies should be conducted with the utmost care to avoid exogenous contamination as previously discussed in this chapter. Equally important, results must be interpreted from a broad microbiological perspective, considering not only the nature of the putative contaminant, but the likelihood of that organism(s) surviving in the pharmaceutical ingredient, excipient, or environment under test. In addition, the growth characteristics of the microorganism should be considered (especially in questions of the growth of filamentous fungi in liquid media).

When results are observed that do not conform to a compendial monograph or other established acceptance criteria, an investigation into the microbial data deviation (MDD) is required. There are generally two distinct reasons for the observation of microbial contamination that does not comply with a target or requirement: There may be either a laboratory error or laboratory environmental conditions that produced an invalid result, or the product contains a level of contamination or specific types of contaminants outside established levels or limits. In either case, laboratory management and, in most cases, the Quality Unit should be notified immediately.

A full and comprehensive evaluation of the laboratory situation surrounding the result should be undertaken. All microbiological conditions or factors that could bring about the observed condition should be fully considered, including the magnitude of the excursion compared to established limits or levels. In addition, an estimate of the variability of the assay may be required in order to determine whether the finding is significant.

The laboratory environment, the protective conditions in place for sampling, historical findings concerning the material under test, and the nature of the material, particularly with regard to microbial survival or proliferation in contact with the material, should be considered in the investigation. In addition, interviews with the laboratory analyst(s) may provide information regarding the actual conduct of the assay that can be valuable in determining the reliability of the result and in determining an appropriate course of action. If laboratory operations are identified as the cause of the nonconforming test outcome, then a corrective action plan should be developed to address the problem(s). Following the approval and implementation of the corrective action plan, the situation should be carefully monitored and the adequacy of the corrective action determined.

If assay results are invalidated on the basis of the discovery of an attributable error, this action must be documented. Laboratories also should have approved procedures for confirmatory testing (retesting), and if necessary, resampling where specific regulatory or compendial guidance does not govern the conduct of an assay investigation.

⟨1118⟩ MONITORING DEVICES—TIME, TEMPERATURE, AND HUMIDITY

INTRODUCTION

This chapter provides background information about the science and technology of temperature and humidity monitoring over time. It describes the available technologies and performance characteristics, and provides recommendations for qualifying performance. The shelf life of a drug product is a function of the temperature and humidity conditions during storage and transportation, as well as the drug product's chemical and physical properties. For this reason, the ability to monitor those conditions is important in the shipping and storage of temperature- and humidity-sensitive drug products. This chapter focuses strictly on supply chain temperature- and humidity-monitoring devices, both electronic and chemical.

The storage and distribution temperatures may be different if justified by appropriate stability studies and as indicated in the labeling. The effects of humidity are typically observed over longer time periods of exposure than are temperature effects due to the barrier to moisture ingress presented by the primary and secondary drug product packaging.

The devices described in this chapter are those most commonly used to monitor the controlled storage and established distribution of drug products following Good Distribution Practices (GDP).[1] The chapter does not address measurement of temperature at extremes, which are temperatures above those that drugs are reasonably expected to experience in the supply chain. The types of devices described are already used in the worldwide distribution of pharmaceuticals and by other similar industries that require temperature control in distribution (for example, the perishable food, blood component, and medical device industries). Devices also may be attached to individual items for the end user (for example, vaccine vials in the World Health Organization (WHO)/UNICEF global immunization program).[2] Appropriate recycling practices should be followed for all devices as required by local regulations.

[1] PDA Technical Report 52 (TR 52) Guidance for Good Distribution Practices (GDPs) For the Pharmaceutical Supply Chain.

[2] World Health Organization (WHO)-UNICEF Policy Statement on the Implementation of Vaccine Vial Monitors: The Role of Vaccine Vial Monitors in Improving Access to Immunization, http://whqlibdoc.who.int/hq/2007/WHO_IVB_07.04_eng.pdf.

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TEMPERATURE-MEASUREMENT DEVICES

Alcohol or Mercury Thermometers

These devices are based on the change in volume of a liquid as a function of temperature. Both types of thermometers can be designed to indicate the maximum and minimum temperatures (see *General Notices, 6.80.30. Temperature Reading Devices* for more information). Historically, these types of thermometers are used in a laboratory setting or in a pharmacy, rather than during supply chain monitoring. Alcohol thermometers can have a precision as good as 0.01°, but they must be quite large to measure temperatures in ranges of more than a few degrees.

Mercury thermometers are typically used in the ranges from 0° to 50° with a precision of about 0.1°. Some local regulations apply to mercury-based thermometers, and many states and local agencies have legislated or developed collection or exchange programs for mercury-containing devices. The U.S. Environmental Protection Agency also issues regulations requiring industry to reduce mercury releases to air and water, and to properly treat and dispose of mercury wastes to avoid potential health hazards.[3] Globally, Health Care Without Harm and WHO are co-leading a Health Care Initiative Products Partnership to reduce demand for mercury-containing devices by at least 70% by 2017 and to shift production to accurate, affordable, and safer nonmercury alternatives.[4]

Both alcohol and mercury thermometers are more fragile than other temperature-measuring devices described in this chapter.

Infrared Devices

This device is used for measuring the infrared (IR) radiant heat from the article whose temperature is being determined, and the IR reading varies as a function of the object's temperature. The advantage of this type of device is that the article may be at some distance from the IR sensor. IR devices may give inaccurate higher or lower temperature readings because of the surface characteristics of packages (e.g., black vs. white surfaces), and they also have the potential for operator error because of incorrect use of the IR reader (improper angle).

Resistance Temperature Detectors

The resistance temperature detector (RTD) is based on the change in electrical resistance of a material as a function of temperature. The precision and accuracy of an RTD depend on the quality of the electronics used to measure the resistance. Although RTDs are among the most stable and accurate temperature sensors, their accuracy may change with the age and temperature of the device because its electronic components are affected by age and use. Although all temperature-measurement devices should be placed on an appropriate calibration program as recommended by the manufacturer or user of the device, this calibration is particularly important for RTDs.

Solid-State Devices

Solid-state devices are based on the effect of temperature on either an integrated circuit (see *Thermistors*) or a micromechanical or microelectrical system. These devices are commonly referred to as data loggers, and can attain high precision and have the advantage of producing a digital output.

Thermistors

A thermistor is a semiconductor device whose resistance varies with temperature. Thermistors are able to detect very small changes in temperature and are accurate over a broad range of temperatures.

Thermocouples

Thermocouples are based on the change in the junction potential of two dissimilar metals as a function of temperature. Many metal pairs can be used, and each pair provides a unique range, accuracy, and precision. Precision and accuracy depend on the quality of the electronics used to measure the voltage across both metals and the type of temperature reference used.

Thermomechanical Devices

Thermomechanical devices are based on the change in length of a solid material as a function of temperature. An example of such a device is a mechanical spring, which expands or contracts as a function of temperature, thus opening and closing an electrical circuit or moving a chart pen. Typical examples are chart recorders used in cold rooms.

[3] http://www.epa.gov/mercury/.
[4] http://www.noharm.org/.

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ELECTRONIC TIME–TEMPERATURE HISTORY RECORDERS

These recorders use one of the electronic temperature-measurement technologies described above and create a record of the temperature history experienced.

Electronic Time–Temperature Indicators

Electronic time–temperature indicators (TTIs) can be designed to alarm after a cold excursion, heat excursion, or after multiple temperature excursions and can provide a visual alarm by a colored light or LCD. The alarm(s) are generally programmable and can display conditions such as date, time, temperature, and duration of the alarm. A certificate of calibration is issued for individual units or lots. Multiple-use devices should have a calibration schedule, but single-use devices can rely on manufacturers' certificates of calibration.

Electronic Temperature-Data Loggers

Electronic temperature-data loggers are recorders that monitor the temperature at programmable intervals and save the temperature history to a peripheral system, such as a personal computer. In addition, data loggers can record humidity using sensors described below. Electronic recorders monitor and save temperature values representative of the cumulative temperature history over a period of time and therefore have the advantage of being able to calculate the mean kinetic temperature (MKT)[5] based on the measurements. Data loggers equipped with transmitting devices (hardwired or radio transmission) can be used to monitor the temperature and humidity of a product while in transit and can download the recorded data when the data loggers arrive at a destination. Data loggers are increasingly required by worldwide ministries of health as part of a standard quality system for GDPs. Based on their communication capabilities, data loggers can be grouped into several different categories.

Radio-Frequency Data Loggers

In addition to data loggers that require a hardwired connection to a base unit or a computer, in recent years companies have adapted wireless (radio-frequency or RF-enabled) temperature and humidity recorders. The effects of radio-frequency identification (RFID) use on biologics have been studied on a variety of blood, blood components, monoclonal antibodies, and vaccines, and have demonstrated no effect.[6] These loggers are integrated with chips capable of wireless RF communications that constitute the RFID sensor tags. The RFID chip inside the tag can be either active, which requires battery power for operation, or passive, which requires a nonzero RF field created by the RFID interrogator host unit (commonly called the reader) in the vicinity of the tag. RFID-enabled sensor tags (temperature and/or humidity) have the added capability of conveying recorded temperature history wirelessly to a host computer or database for seamless downstream processing. Multiple passive and active standards exist to control the communication between the tag and the host unit, including ISO-18000-6C,[7] ISO-18000-7,[8] ZigBee,[9] IEEE 802.11,[10] and many proprietary standards.

When choosing between active and passive technologies, one needs to know that active technologies typically have extended communication range and memory capabilities at the expense of a higher price. Currently, reading ranges extend to 100 m and with repeaters longer distances can be achieved. Whether the communications circuitry is passive or active, these RF loggers still are electronic temperature recorders, which means their sensor circuitry uses external power from batteries or other sources.

A completely passive wireless RFID tag with an antenna capable of functioning as a sensor has been developed. The tag uses resonant antenna structures of RFID tags that are coated with specific sensor films. The passive wireless RFID tags act like analog sensors that, when interrogated by a wireless reader, show the instantaneous temperature. The film changes the antenna's reflection characteristics based on the monitored environmental variable (such as temperature and/or humidity), which then is decoded by the reader. The sensor film can be designed to work with different variables such as temperature, humidity, and various gas and chemical vapors. Although they lack some of the memory functionality included in electronic recorders, passive wireless sensors are relatively cost effective compared to data loggers and can be considered for item-level applications.

CHEMICAL TEMPERATURE INDICATORS

Chemical temperature indicators are relatively cost effective compared to electronic data loggers and can be considered for item-level applications There are two basic types of chemical temperature indicators: 1) a threshold indicator that responds at a specific temperature and 2) a TTI that responds to cumulative heat exposure.

[5] The Use of Mean Kinetic Temperature (MKT) in the Handling, Storage, and Distribution of Temperature Sensitive Molecules. R. H. Seevers, J. Hofer, P. Haber, D. A. Ulrich, R. Bishara. Pharmaceutical Outsourcing, May/June, 30–37, 2009.

[6] Effects of Radio Frequency Identification-Related Radiation on In Vitro Biologics. I. Uysal, C. Hohberger, R. S. Rasmussen, D. A. Ulrich, J. P. Emond, A. Gutierrez. PDA Journal of Pharmaceutical Science and Technology, Vol. 66(4), July/Aug, 333–345, 2012.

[7] ISO/IEC 18000-6:2010–ISO/IEC 18000-6:2010—Information technology– Radio frequency identification for item management–Part 6: Parameters for air interface communications at 860 MHz to 960 MHz.

[8] ISO/IEC 18000-7:2009—Information technology–Radio frequency identification for item management–Part 7: Parameters for active air interface communications at 433 MHz.

[9] IEEE 802.15.4—Wireless Medium Access Control (MAC) and Physical Layer (PHY) Specifications for Low-Rate Wireless Personal Area Networks (LR-WPANs), 2003.

[10] IEEE 802.11—Wireless Local Area Networks (LANs), 2007.

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Chemical Temperature Threshold Indicators

These indicators, sometimes referred to as critical temperature indicators, are based on a phase change or chemical reaction that occurs as a function of temperature. Examples include liquid crystals, waxes, polymers, and lacquers that change phase, and thereby their appearance, as a function of temperature. Chemical temperature threshold indicators are reversible or irreversible and are suitable for high or low temperatures. Temperature threshold indicators do not include any specified time delay to show a response and typically are single-use devices. These indicators provide a signal only when exposed to temperatures higher than (ascending indicator) or lower than (descending indicator) a predetermined threshold temperature.

Ascending-Temperature Threshold Indicators—Ascending-temperature threshold indicators are supplied as self-adhesive labels or cards and are normally composed of a heat-fusible compound that melts at the critical temperature. Melting of the compound gives rise to a color change or color development. Other types are provided as inks, lacquers, pellets, or crayons. Ascending-temperature threshold indicators are available from 0° to more than 200° and as many as 10 temperatures on a single unit. Some ascending-temperature threshold indicators used to monitor frozen or refrigerated temperatures require an activation step (such as a pull tab or a reservoir that is ruptured by pressure).

Descending-Temperature Threshold Indicators—Descending-temperature threshold indicators show a response when exposed to temperatures below a threshold. These indicators do not include a specific time delay to show a response, and the response is typically caused by the time required for solidification of a liquid at the threshold temperature. Solidification of a liquid causes a visual change in the indicator. Examples include: 1) the expansion of the liquid to crack an ampule and release a colorant, 2) contraction of the liquid to mix components to develop color, or 3) aggregation of colloidal particles to change color.

Chemical Time–Temperature Indicators

These indicators, sometimes referred to as time–temperature integrators (TTIs), include systems in which a reaction rate or diffusion process is used to estimate a temperature equivalent integrated over time. Thus, TTIs provide a measure of accumulated heat rather than instantaneous temperature such as a spike or critical threshold (discussed above). The reactions generally are irreversible—once a color change, color development, or diffusion process has taken place, exposure to low temperatures will not restore the indicator to its original state, but lower temperatures (refrigeration) will slow the color change. The accuracy and precision of these indicators depend, to some extent, on human interpretation. Some versions of chemical indicators have been prepared in a bar code format and can be read with bar code readers. Other developments include reading a chemical indicator with an imaging device such as a camera in a smart phone.

TTIs do not directly reflect the status of the drugs to which they are attached. In actual practice, the characteristics for degradation of a particular drug are known from accelerated and real-time stability studies that follow internationally accepted guidelines such as PDA TR 53[11] to guide selection of a suitable TTI.[12] The activation energy of the TTI is not required to exactly match the activation energy of the degradation of the drug being monitored, and the latter, in fact, may not be known precisely. Therefore, the TTI should be chosen to provide an early warning if the drug is exposed to an excessive heat load before the expiration date.

An important characteristic of chemical TTIs is the precision with which the end point can be determined. For TTIs that change color, a reference color normally brackets the active portion of the TTI to show the end point color, which simplifies TTI interpretation. Accuracy can vary widely with the control and quality of the manufacturing process. Some TTIs are manufactured by procedures that comply with Quality System Regulations for Medical Devices. As discussed below in *Calibration of Temperature- and Humidity-Monitoring Devices*, it is not possible to calibrate any individual single-use device because the test is, by the nature of the TTI, necessarily destructive. This is analogous to any pharmaceutical product because each dose cannot be calibrated or validated, but validated processes should be used in the manufacturing process.

The two types of TTIs are partial-history indicators and full-history indicators. Partial-history TTIs provide a time- and temperature-dependent response when the temperature exceeds a predetermined value. A partial-history indicator normally is composed of a dyed, heat-fusible compound that diffuses along a porous strip or wick when the temperature exceeds the melting point of the compound. The diffusion process of the compound down the wick is temperature dependent, and therefore the partial-history TTI provides a time and temperature response above the melting point of the compound. Migration of the compound down the wick stops when the indicator is moved to a lower temperature at which point the compound solidifies. These TTIs normally have one or more viewing windows to monitor the length the dyed compound has traveled along the wick. Some indicators are activated by removing a barrier film that separates the dyed compound from the wick or rupturing a reservoir that contains the dyed compound. Other indicators do not require activation and must be stored below the melting point of the compound before use. These partial-history TTIs can have durations (service life) anywhere from several hours to several years. Full-history TTIs provide a continuous response to temperature. They change color or physical appearance as a result of exposure to time, and the rate of change increases with temperature so they are sensitive to cumulative heat exposure. Full-history TTIs are responsive to MKT (Ref 1079) and typically are single use, irreversible, and disposable because once the color changes it will not revert to the original color.

Chemical–Physical Time–Temperature Indicators

This type of TTI is based on a temperature-dependent diffusion or chemical reaction process. It consists of a pressure-sensitive tape device that is composed of an indicator tape and an activator tape. In one example, the indicator tape contains a dye precursor dispersed in a polymer carrier. The activator is incorporated into an adhesive on the activator tape. Laminating the activator tape over the indicator tape causes activation. A color change or readable message occurs as the activator migrates

[11] PDA Technical Report 53 (TR 53) Guidance for Industry: Stability Testing to Support Distribution of New Drug Products, https://store.pda.org.
[12] ASTM F1416–96 (2008) Standard Guide for Selection of Time Temperature Indicators.

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into the indicator as a function of temperature and time. Other approaches to develop color changes include the use of a pH indicator or the etching of aluminum by the activator tape.

Chemical Polymerization–Based Time–Temperature Indicators

This type of TTI uses a solid-state polymerization process in which a color develops intensity as a function of time and temperature. The color evolution is caused by the polymerization of a colorless monomer to a highly colored polymer. These TTIs can be applied by a print process that permits direct integration into a product label or stand-alone label. Because this type of TTI does not require activation, it must be shipped from the manufacturer on dry ice or under frozen conditions and stored at temperatures according to the manufacturer's instructions, normally below –24° before use. Chemical polymerization–based TTIs can be designed to reach the end point as quickly as weeks at refrigerated temperature or as long as years at controlled room temperature.

Chemical Enzyme–Based Time–Temperature Indicators

This type of TTI uses an enzyme-catalyzed color-generating reaction that occurs as a function of time and temperature. The color change is caused by an enzyme reacting with a substrate, accompanied by a change in pH. The enzyme and substrate are in separate solutions in adjacent compartments. Breaking the barrier between the two compartments and mixing the two solutions activates the TTI.

Chemical-Organic Pigment–Based Time–Temperature Indicators

This type of TTI uses an organic pigment that is activated by exposure to ultraviolet light to develop a dark blue starting color. A filter is then placed over the label to protect it from deliberate or accidental reactivation. The colored pigment fades over time as a function of temperature.

RELATIVE HUMIDITY MEASUREMENT TECHNOLOGIES

Relative humidity is the ratio of the partial pressure of water vapor in air to the vapor pressure of saturated air at a given temperature. In other words, the relative humidity is the amount of water vapor present, divided by the theoretical amount of moisture that could be held by that volume of air at a given temperature. Extensive tables of relative humidity data are available. Devices for measuring relative humidity are called hygrometers. Several different technologies exist for measuring relative humidity.

Sling Psychrometer

The simplest type of hygrometer is based on the temperature difference observed between two identical thermometers, one ordinary and one with a wet cloth wick over its bulb. The two thermometers are whirled at the end of a chain, and the evaporation of water from the wick cools (based on evaporative cooling) the wet-bulb thermometer. The temperature difference between the wet and dry thermometers then is compared to a table specific to that psychrometer based on dry-bulb temperature, and the relative humidity is determined.

Hair Hygrometer

This type of device is based on the fact that the length of a synthetic or human hair increases as a function of the relative humidity. This change is used to move an indicator or affect a strain gauge. A hair hygrometer can be accurate to ±3%, but it is unable to respond to rapid changes in humidity and loses accuracy at very high or very low levels of relative humidity.

Infrared Hygrometer

This type of hygrometer determines relative humidity by comparing the absorption of two different wavelengths of IR radiation through air. One wavelength is absorbed by water vapor and the other is not. This type of hygrometer can accurately measure relative humidity in large or small volumes of air. It is sensitive to rapid changes of humidity and can be integrated with an electronic data-handling system.

Dew Point Hygrometer

This type of device uses a chilled mirror to determine the dew point of an air sample. The dew point is the temperature at which water vapor in the air begins to condense; that is, the temperature at which the relative humidity is 100%. The relative humidity can be calculated from this measurement and an accurate measurement of the ambient temperature. The dew point hygrometer is the standard against which most commercially available instruments are calibrated.

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Capacitive Thin-Film Hygrometer

The principle of this type of hygrometer is that the dielectric of a nonconductive polymer changes in direct proportion to the relative humidity. This change is measured as a change in capacitance. This type of hygrometer is accurate to ±3%.

Resistive Thin-Film Hygrometer

This type of hygrometer is similar to the capacitive thin-film type because it uses the effect of changing relative humidity on an electric circuit. In the resistive thin-film hygrometer the sensor is an organic polymer whose electrical resistance changes in logarithmic proportion to the relative humidity. This type of hygrometer is accurate to ±5%.

CALIBRATION OF TEMPERATURE- AND HUMIDITY-MONITORING DEVICES

Thermometers and hygrometers that are used to provide data about the temperature and humidity exposure of a product must be suitable for their intended use. Specifically, they must be appropriately calibrated. A calibration program assures the user of the monitoring device that the device has been tested before use either by the manufacturer or the user to assess the suitability for its intended use. Calibrations should be performed with appropriate frequencies to support ongoing use. Monitors used in manufacturing, storage, and transport of drug products should be properly qualified by their users to ensure that the monitors have been received and maintained in proper working order. It is acceptable to use the calibration performed by the device's manufacturer based on the certificate of calibration and expiration date.

For temperature- and humidity-monitoring devices, measurement accuracy refers to the closeness of the value obtained with a particular device and the true value of the object or environment under measurement. In practice, this is determined by comparison with a device that has been calibrated against a standard that is obtained from or is traceable to the National Institute of Standards and Technology or a comparable national metrology organization.

Any monitor takes time to respond to a change in the temperature or humidity. Measurement responsiveness typically is defined in a device's specifications for its operating range. Different data recording intervals are appropriate for different monitoring applications and should be based on supply chain length (for example, transportation via ocean may require 30-min intervals, but 15-min intervals may be suitable for air transport). Most commonly, time accuracy is expressed as a ± percentage of total duration of the recording period. For pharmaceutical applications, a ±0.5% time accuracy is adequate.

Single-use electronic and chemical indicators should follow Good Manufacturing Practices with appropriate testing controls. Electronic indicators require proper calibration. Single-use indicator performance can be qualified by the supply chain user by sampling and testing of multiple production lots. For TTIs that calculate MKT, the performance of a batch can be assessed statistically by subjecting an appropriately sized sample to elevated temperature conditions for a set period of time and observing the results. Manufacturers should adopt appropriate acceptance criteria. It is acceptable to use the release test performed by the manufacturer of the indicator (based on the certificate of calibration or the certificate of analysis and the expiration date) in lieu of calibration or qualification.

THE USE OF HISTORICAL TEMPERATURE DATA

Although historical geographic and seasonal trends may be used as a planning tool in selecting among the types of temperature- and humidity-monitoring devices, outside ambient temperatures are not necessarily reliable indicators of the temperatures experienced by different items in the distribution chain. For example, studies have reported important departures from ambient temperatures on summer days for mailboxes, trucks, and warehouses.[13] Therefore, using lane-specific temperature monitoring is beneficial when manufacturers and shippers develop an ambient profile and can be a valuable consideration for a risk-based approach to maintain product quality.[14]

A drug product's quality (identity, strength, and purity) may be notably influenced by variations in temperature and humidity over the course of its shelf life, so manufacturers should appropriately monitor those environmental conditions. Pharmaceutical manufacturers perform stability testing to carefully evaluate the effects of temperature and humidity on their products. The packaging, shelf life, and storage and transportation conditions recommended for a product are chosen based on the results of these stability studies. Temperature effects can happen rapidly; therefore, temperature monitoring should be implemented on a risk-based approach taking the product stability, distribution route, mode of transportation and potential risks that may compromise the quality of the product into account. Relative humidity effects occur over a much longer time frame; humidity monitoring can be omitted when the drug product is sufficiently protected by the primary container proven by sound stability studies. Humidity monitoring is recommended when special environmental restrictions concerning the humidity are defined for the drug product.

[13] Okeke, C.C. Medwick, T., Bailey, L.C., and Grady L.T. Temperature and Humidity Conditions During Shipment in International Commerce, *PF* 25(2) Mar.–Apr. 1999.
[14] ISTA 7E Standard, http://www.ista.org.

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⟨1120⟩ RAMAN SPECTROSCOPY

INTRODUCTION

Raman is a vibrational spectroscopic technique and is therefore related to infrared (IR) and near-infrared (NIR) spectroscopy. The Raman effect itself arises as a result of a change in the polarizability of molecular bonds during a given vibrational mode and is measured as inelastically scattered radiation.

A Raman spectrum is generated by exciting the sample of interest to a virtual state with a monochromatic source, typically a laser. Light elastically scattered (no change in wavelength) is known as Rayleigh scatter and is not of interest in Raman spectrometry, except for marking the laser wavelength. However, if the sample relaxes to a vibrational energy level that differs from the initial state, the scattered radiation is shifted in energy. This shift is commensurate with the energy difference between the initial and final vibrational states. This "inelastically scattered" light is referred to as Raman scatter. Only about one in 10^6–10^8 photons incident on the sample undergoes Raman scattering. Thus lasers are employed in Raman spectrometers. If the Raman-scattered photon is of lower energy, it is referred to as Stokes scattering. If it is of higher energy, it is referred to as anti-Stokes scattering. In practice, nearly all analytically useful Raman measurements make use of Stokes-shifted Raman scatter.

The appearance of a Raman spectrum is much like an infrared spectrum plotted linearly in absorbance. The intensities, or the number of Raman photons counted, are plotted against the shifted energies. The x-axis is generally labeled "Raman Shift/cm^{-1}" or "Wavenumber/cm^{-1}". The Raman shift is usually expressed in wavenumber and represents the difference in the absolute wavenumber of the peak and the laser wavenumber. The spectrum is interpreted in the same manner as the corresponding mid-infrared spectrum. The positions of the (Raman shifted) wavenumbers for a given vibrational mode are identical to the wavenumbers of the corresponding bands in an IR absorption spectrum. However, the stronger peaks in a Raman spectrum are often weak in an IR spectrum, and vice versa. Thus the two spectroscopic techniques are often said to be complementary.

Raman spectroscopy is advantageous because quick and accurate measurements can often be made without destroying the sample (solid, semisolid, liquid or, less frequently, gas) and with minimal or no sample preparation. The Raman spectrum contains information on fundamental vibrational modes of the sample that can yield both sample and process understanding. The signal is typically in the visible or NIR range, allowing efficient coupling to fiber optics. This also means that a signal can be obtained from any medium transparent to the laser light; examples are glass, plastics, or samples in aqueous media. In addition, because Raman spectra are ordinarily excited with visible or NIR radiation, standard glass/quartz optics may be used. From an instrumental point of view, modern systems are easy to use, provide fast analysis times (seconds to several minutes), and are reliable. However, the danger of using high-powered lasers must be recognized, especially when their wavelengths are in the NIR and, therefore, not visible to the eye. Fiber-optic probes should be used with caution and with reference to appropriate government regulations regarding lasers and laser classes.

In addition to "normal" Raman spectroscopy, there are several more specialized Raman techniques. These include resonance Raman (RR), surface-enhanced Raman spectroscopy (SERS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), Raman gain or loss spectroscopy, and hyper-Raman spectroscopy. These techniques are not widely employed in pharmaceutical laboratories, and are not addressed in this general information chapter.

QUALITATIVE AND QUANTITATIVE RAMAN MEASUREMENTS

There are two general classes of measurements that are commonly performed by Raman spectrometry: qualitative and quantitative.

Qualitative Raman Measurements

Qualitative Raman measurements yield spectral information about the functional groups that are present in a sample. Because the Raman spectrum is specific for a given compound, qualitative Raman measurements can be used as a compendial ID test, as well as for structural elucidation.

Quantitative Raman Measurements

For instruments equipped with a detector that measures optical power (such as Fourier transform [FT]-Raman spectrometers), quantitative Raman measurements utilize the following relationship between signal, S_v, at a given wavenumber, v, and the concentration of an analyte, C:

$$S_v = K\sigma_v(\nu_L - \nu\beta)^4 P_0 C$$

in which K is a constant that depends on laser beam diameter, collection optics, sample volume, and temperature; σ_v is the Raman cross section of the particular vibrational mode; ν_L is the laser wavenumber; ν_β is the wavenumber of the vibrational mode; and P_0 is the laser power. The Raman cross section, σ_v, is characteristic of the nature of the particular vibrational mode. The sample volume is defined by size of the focus of the laser beam at the sample, the optic being used for focusing, and the optical properties of the sample itself. Spot sizes at the sample can range from less than 1 µm for a microprobe to 6 mm for a large area sample system. For Raman spectrometers that measure the number of photons per second (such as change-coupled device [CCD]-Raman spectrometers) the corresponding equation is:

$$S_v = K\sigma_v\nu_L(\nu_L - \nu\beta)^3 P_0 C$$

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From the above equations, it is apparent that peak signal is directly proportional to concentration. It is this relationship that is the basis for the majority of quantitative Raman applications.

FACTORS AFFECTING QUANTIFICATION

Sample-Based Factors

The most important sample-based factors that deleteriously affect quantitative Raman spectrometry are fluorescence, sample heating, absorption by the matrix or the sample itself, and the effect of polarization. If the sample matrix includes fluorescent compounds, the measured signal will usually contain a contribution from fluorescence. Fluorescence will be observed only if the laser excitation wavelength overlaps with an absorption band of a fluorescent compound. Fluorescence is typically observed as a broad sloping background underlying the Raman spectrum. Fluorescence can cause both a baseline offset and reduced signal-to-noise ratio. The wavelength range and intensity of the fluorescence is dependent on the chemical composition of the fluorescent material. Because fluorescence is generally a much more efficient process than Raman scattering, even very minor amounts of fluorescent impurities can lead to significant degradation of the Raman signal. Fluorescence can be reduced by using longer wavelength excitation sources such as 785 nm or 1064 nm. However, it should be remembered that the strength of the Raman signal is proportional to $(\nu_L - \nu_\beta)^4$, so the advantage of using a long-wavelength excitation laser to minimize fluorescence is at least partially offset by the reduced strength of the Raman signal. The greatest signal-to-noise ratio will be obtained by balancing fluorescence rejection, signal strength, and detector response.

Fluorescence in solids can sometimes be mitigated by exposing the sample to the laser radiation for a period of time before measurement. This process is called photobleaching, and operates by degrading the highly absorbing species. Photobleaching is less effective in liquids, where the sample is mobile, or if the amount of fluorescent material is more than a trace.

Sample heating by the laser source can cause a variety of effects, such as physical form change (melting), polymorph conversion, or sample burning. The chance for sample heating is greatest when the spot size at the sample is the smallest, i.e., when a microprobe is being used. This is usually an issue for colored, highly absorbing species, or very small particles that have low heat transfer. The effects of sample heating are usually observable either as changes in the Raman spectrum over time or by visual inspection of the sample. Besides decreasing the laser flux, a variety of methods can be employed to diminish laser-induced heating, such as moving the sample or laser during the measurement or improving the heat transfer from the sample with thermal contact or liquid immersion.

Absorption of the Raman signal by the matrix or the sample itself can also occur. This problem is more prevalent with long-wavelength FT-Raman systems where the Raman signal can overlap with an NIR overtone absorption. This effect will be dependent on the optics of the system as well as on the sample presentation. Associated with this effect is variability from scattering in solids as a result of packing and particle-size differences. The magnitude of all of these effects, however, is typically less severe than in NIR because of the limited depth of penetration and the relatively narrower wavelength region sampled in Raman spectroscopy.

Finally, it should be recognized that laser radiation is polarized and the Raman spectra of crystalline materials and other oriented samples can differ significantly depending on the way that the sample is mounted. If the Raman spectrometer is capable of producing linearly polarized radiation at the sample then a polarization scrambler is recommended for routine sample analysis.

Sampling Factors

Raman spectroscopy is a zero-background technique, in that the signal at the detector is expected to be zero in the absence of a sample. This situation can be contrasted with absorption spectrometry, where the signal at the detector is at a maximum in the absence of a sample. Zero-background techniques are inherently sensitive because small changes in sample concentration lead to proportionate changes in the signal level. The instrument will also be sensitive to other sources of light that can cause sample-to-sample variations in the measured signal level. In addition, a large background signal caused by fluorescence will lead to an increased noise level (photon shot noise). Thus it may be very difficult to use the absolute Raman signal for direct determination of an analyte. Other potential sources of variation are changes in the sample opacity and heterogeneity, changes in the laser power at the sample, and changes in optical collection geometry or sample position. These effects can be minimized by sampling in a reproducible, representative manner. Careful design of the instrumentation can reduce these effects but they cannot be eliminated entirely.

Use of an internal reference standard is the most common and robust method of eliminating variations caused by absolute intensity fluctuations. There are several choices for this approach. An internal standard can be deliberately added, and isolated peaks from this standard can be employed; or a band due to a moiety such as an aromatic ring, the Raman cross-section of which does not change with the way the sample is prepared, can also be used. For solution spectra, an isolated solvent band can be employed because the solvent will remain relatively unchanged from sample to sample. Also, in a formulation, an excipient peak can be used if it is in substantial excess compared to the analyte. The entire spectrum can also be used as a reference, with the assumption that laser and sample-orientation changes will affect the entire spectrum equally.

A second important sampling-based factor to consider is spectral contamination. Raman scattering is a weak effect that can be masked by a number of external sources. Common contamination sources include sample-holder artifacts (container or substrate) and ambient light. Typically, these issues can be identified and resolved by careful experimentation.

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APPARATUS

Components

All modern Raman measurements involve irradiating a sample with a laser, collecting the scattered radiation, rejecting the Rayleigh-scattered light, differentiating the Raman photons by wavelength, and detecting the resulting Raman spectrum. All commercial Raman instruments therefore share the following common features to perform these functions:

1. Excitation source (laser)
2. Sampling device
3. Device to filter/reject light scattered at the laser wavelength
4. Wavelength processing unit
5. Detector and electronics

EXCITATION SOURCE (LASER)

Table 1 identifies several common lasers used for pharmaceutical applications or Raman spectrometry. UV lasers have also been used for specialized applications but have various drawbacks that limit their utility for general analytical measurements. As more applications for UV lasers are described, it is likely that they may become more common for Raman spectrometry.

Table 1. Lasers Used in Pharmaceutical Applications

Laser λ, nm (nearest whole number)	Type	Typical Power at Laser	Wavelength Range, nm (Stokes Region, 100 cm^{-1} to 3000 cm^{-1} shift)	Comments
NIR Lasers				
1064	Solid state (Nd:YAG)	Up to 3 W	1075–1563	Commonly used in Fourier transform instruments
830	Diode	Up to 300 mW	827–980	Typically limited to 2000 cm^{-1}; Raman shift because of CCD spectral response; less common than the other lasers
785	Diode	Up to 500 mW	791–1027	Most widely used dispersive Raman laser
Visible Lasers				
632.8	He–Ne	Up to 500 mW	637–781	Relatively small fluorescence risk
532	Doubled (Nd:YAG)	Up to 1 W	535–632.8	High fluorescence risk
514.5	Ar+	Up to 1 W	517–608	High fluorescence risk
488–632.8	Ar+	Up to 1 W	490–572	High fluorescence risk

SAMPLING DEVICE

Several sampling arrangements are possible, including direct optical interfaces, microscopes, fiber optic-based probes (either noncontact or immersion optics), and sample chambers (including specialty sample holders and automated sample changers). The sampling optics can also be designed to obtain the polarization-dependent Raman spectrum, which often contains additional information. Selection of the sampling device will often be dictated by the analyte and sample. However, considerations such as sampling volume, speed of the measurement, laser safety, and reproducibility of sample presentation should be evaluated to optimize the sampling device for any given application.

FILTERING DEVICE

The intensity of scattered light at the laser wavelength (Rayleigh) is many orders of magnitude greater than the Raman signal and must be rejected prior to the detector. Notch filters are almost universally used for this purpose and provide excellent rejection and stability combined with small size. The traditional use of multistage monochromators for this purpose, although still viable, is now rare. In addition, various filters or physical barriers to shield the sample from external radiation sources (e.g., room lights, laser plasma lines) may be required depending on the collection geometry of the instrument.

WAVELENGTH PROCESSING UNIT

The wavelength scale may be encoded by either a scanning monochromator, a grating polychromator (in CCD-Raman spectrometers) or a two-beam interferometer (in FT-Raman spectrometers). A discussion of the specific benefits and drawbacks of each of the dispersive designs compared to the FT instrument is beyond the scope of this chapter. Any properly qualified instruments should be suitable for qualitative measurements. However, care must be taken when selecting an instrument for quantitative measurements, as dispersion and response linearity might not be uniform across the full spectral range.

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DETECTOR

The silicon-based CCD array is the most common detector for dispersive instruments. The cooled array detector allows measurements over the spectral range from 4500 to 100 cm^{-1} Raman shift with low noise when most visible lasers, such as frequency-doubled neodymium-doped yttrium–aluminum–garnet (Nd:YAG) (532 nm) or helium–neon (632.8 nm) lasers, are used. When a 785-nm diode laser is used, the wavelength range is reduced to about 3100 to 100 cm^{-1}. The most commonly used CCD has its peak wavelength responsivity when matched to the commonly used 632.8-nm He–Ne gas laser or 785-nm diode laser. FT instruments typically use single-channel germanium or indium–gallium–arsenide (InGaAs) detectors responsive in the NIR to match the 1064-nm excitation of a Nd:YAG laser.

Calibration

Raman instrument calibration involves three components: primary wavelength (x-axis), laser wavelength, and intensity (y-axis).

PRIMARY WAVELENGTH (X-AXIS)

In the case of FT-Raman instruments, primary wavelength-axis calibration is maintained, at least to a first approximation, with an internal He–Ne laser. Most dispersive instruments utilize atomic emission lamps for primary wavelength-axis calibration. In all instruments suitable for analytical Raman measurements, the vendor will offer a procedure of x-axis calibration that can be performed by the user. For dispersive Raman instruments, a calibration based on multiple atomic emission lines is preferred. The validity of this calibration approach can be verified subsequent to laser wavelength calibration by using a suitable Raman shift standard. For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.[1]

LASER WAVELENGTH

Laser wavelength variation can impact both the wavelength precision and the photometric (signal) precision of a given instrument. Even the most stable current lasers can vary slightly in their measured wavelength output. The laser wavelength must therefore be confirmed to ensure that the Raman shift positions are accurate for both FT-Raman or dispersive Raman instruments. A reference Raman shift standard material such as those outlined in ASTM E1840-96 (2002)[1] or other suitably verified materials can be utilized for this purpose. [NOTE—Reliable Raman shift standard values for frequently used liquid and solid reagents, required for wavenumber calibration of Raman spectrometers, are provided in the ASTM Standard Guide cited. These values can be used in addition to the highly accurate and precise low-pressure arc lamp emission lines that are also available for use in Raman instrument calibration.] Spectrometric grade material can be purchased from appropriate suppliers for this use. Certain instruments may use an internal Raman standard separate from the primary optical path. External calibration devices exactly reproduce the optical path taken by the scattered radiation. [NOTE—When chemical standards are used, care must be taken to avoid contamination and to confirm standard stability.]

Unless the instrument is of a continuous calibration type, the primary wavelength axis calibration should be performed, as per vendor procedures, just prior to measuring the laser wavelength. For external calibration, the Raman shift standard should be placed at the sample location and measured using appropriate acquisition parameters. The peak center of a strong, well-resolved band in the spectral region of interest should be evaluated. The position can be assessed manually or with a suitable, valid peak-picking algorithm. The software provided by the vendor might measure the laser wavelength and adjust the laser wavelength appropriately so that this peak is at the proper position. If the vendor does not provide this functionality, the laser wavelength should be adjusted manually. Depending on the type of laser, the laser wavelength can vary with temperature, current, and voltage. Wavelength tolerances can vary depending on the specific application.

SIGNAL LEVEL (Y-AXIS)

Calibration of the photometric axis can be critical for successful quantification by using certain analytical methods (chemometrics) and method transfer between instruments. Both FT-Raman and dispersive Raman spectrometers should undergo similar calibration procedures. The tolerance of photometric precision acceptable for a given measurement should be assessed during the method development stage.

To calibrate the photometric response of a Raman instrument, a broad-band emission source should be used. There are two accepted methods. *Method A* utilizes a tungsten white light source.[2] The output power of such sources is traceable to the National Metrology Institute (NMI). In the United Kingdom, the National Physical Laboratory also provides calibrated light bulbs. Several other vendors also provide NIST-traceable irradiance calibration standards. This method is applicable to all common laser excitation wavelengths listed in *Table 1*. In *Method B,* NIST standard reference materials (SRMs) are utilized.[3] Several doped-glass fluorescence standards are currently available.

Method A—The source should be placed at the sample location with the laser off and the response of the detector measured (using parameters appropriate for the instrument). The output for the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should

[1] ASTM E1840-96 (2002) *Standard Guide for Raman Shift Standards for Spectrometer Calibration,* ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA, USA 19428-2959.

[2] NIST-traceable tungsten white light source statement: While the calibration of the Raman frequency (or Raman shift, cm^{-1}) axis using pure materials and an existing ASTM standard is well accepted, techniques for calibration of the Raman intensity axis are not. Intensity calibrations of Raman spectra can be accomplished with certified white light sources.

[3] NIST SRM 2241: Ray KG, McCreery RL. Raman intensity correction standard for systems operating with 785-nm excitation. *Appl. Spectrosc.* 1997, 51, 108–116.

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be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach.

Method B—The fluorescence standard should be placed at the sample location. With the laser on, a spectrum of the SRM should be obtained (using parameters appropriate for the instrument). The output of the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach. [NOTE—*Method B* is currently appropriate for systems with 785-nm (SRM 2241), 532-nm (SRM 2242), and both 514.5-nm and 488-nm (SRM 2243) laser excitation. NIST is currently developing other SRMs that will be wavelength-specific for 1064-nm (SRM 2244) and 632.8-nm excitation (expected to be available in 2006).]

EXTERNAL CALIBRATION

Detailed functional validation employing external reference standards is recommended to demonstrate instrumental suitability for laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality control procedures; rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis or purpose. For instruments installed in a process location or in a reactor where positioning of an external standard routinely is not possible, including those instruments that employ an internal calibration approach, the relative performance of an internal versus an external calibration approach should be periodically checked. The purpose of this test is to check for changes in components that might not be included in the internal calibration method (process lens, fiber-optic probe, etc.), e.g., photometric calibration of the optical system.

QUALIFICATION AND VERIFICATION OF RAMAN SPECTROMETERS

The suitability of a specific instrument for a given method is ensured by a thorough technology-suitability evaluation for the application; a routine, periodic instrument operational qualification; and the more frequent performance verification (see *Definition of Terms and Symbols*). The purpose of the technology-suitability evaluation is to ensure that the technology proposed is suitable for the intended application. The purpose of the instrument qualification is to ensure that the instrument to be used is suitable for its intended application and, when requalified periodically, continues to function properly over extended time periods. When the device is used for a specific qualitative or quantitative analysis, regular performance verifications are made. Because there are many different approaches to measuring Raman spectra, instrument operational qualification and performance verification often employ external standards that can be used on any instrument. As with any spectrometric device, a Raman instrument needs to be qualified for both wavenumber (x-axis and shift from the excitation source) and photometric (signal axis) precision.

In performance verification, a quality-of-fit to an initial scan or group of scans (often referred to in nonscanning instruments as an accumulation) included in the instrumental qualification can be employed. In such an analysis, it is assumed that reference standard spectra collected on a new or a newly repaired, properly operating instrument represent the best available spectra. Comparison of spectra taken over time on identical reference standards (either the original standard or identical new standards, if stability of the reference standards is a concern) forms the basis for evaluating the long-term stability of a Raman measurement system.

Frequency of Testing

Instrumental qualification is performed at designated intervals or following a repair or significant optical reconfiguration, such as the replacement of the laser, the detector or the notch or edge filters. Full instrument requalification might not be necessary when changing between sampling accessories such as a microprobe, a sample compartment, or a fixed fiber-optic probe. Performance verification tests may be sufficient in these cases; instrument-specific guidance from the vendor on qualification requirements should be followed. Tests include wavelength (x-axis and shift from the excitation source) and photometric (signal axis) precision. Instrument qualification tests require that specific application-dependent tolerances be met.

Performance verification is carried out on the instrument configured for the analytical measurements and is performed more frequently than instrument qualification. Performance verification includes measurement of the wavelength uncertainty and intensity-scale precision. Wavelength precision and intensity-scale precision tests may be needed prior to any data collection on a given day. Performance is verified by matching the current spectra to those collected during the previous instrument qualification.

Instrument Operational Qualification

It is important to note that the acceptance specifications given in both the *Instrument Operational Qualification* and *Performance Qualification* sections are applicable for general use; specifications for particular instruments and applications can vary depending on the analysis method used and the desired accuracy of the final result. ASTM standard reference materials are also specified, with the understanding that under some circumstances (specifically remote on-line applications) calibration using one of these materials may be impractical, and other suitably verified materials can be employed. At this juncture it is important to note that specific parameters such as spectrometer noise, limits of detection (LOD), limits of quantification (LOQ),

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and acceptable spectral bandwidth for any given application should be included as part of the analytical method development. Specific values for tests such as spectrometer noise and bandwidth will be dependent on the instrument chosen and the purpose required. As a result, specific instrument tests for these parameters are not dictated in this information chapter.

WAVELENGTH (X-AXIS) ACCURACY

It is important to ensure the accuracy of the wavelength axis via calibration to maintain the integrity of Raman peak positions. Wavelength calibration of a Raman spectrometer consists of two parts: primary wavelength axis and laser wavelength calibration. After both the primary wavelength axis and the laser wavelength are calibrated, instrument wavelength uncertainty can be determined. This can be accomplished using a Raman shift standard such as the ASTM shift standards or other suitably verified material. Selection of a standard with bands present across the full Raman spectral range is recommended so that instrument wavelength uncertainty can be evaluated at multiple locations within the spectrum. The tolerance of wavelength precision that is required for a given measurement should be assessed during the method-development stage. [NOTE—For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.]

PHOTOMETRIC PRECISION

Laser variation in terms of the total emitted photons occurring between two measurements can give rise to changes in the photometric precision of the instrument. Unfortunately, it is very difficult to separate changes in the photometric response associated with variations in the total emitted laser photons from the sample- and sampling-induced perturbations. This is one of the reasons why absolute Raman measurements are strongly discouraged and why the photometric precision specification is set relatively loosely. The tolerance of photometric precision required for a given measurement should be assessed during the method-development stage.

PERFORMANCE QUALIFICATION

The objective of performance qualification is to ensure that the instrument is performing within specified limits with respect to wavelength precision, photometric axis precision, and sensitivity. In certain cases when the instrument has been set up for a specific measurement (for example, installed in a process reactor), it might no longer be possible or desirable to measure the wavelength and photometric (signal) qualification reference standards identified above. Provided instrument operational qualification has shown that the equipment is fit for use, a single external performance verification standard can be used to reverify function on a continuing basis (for example, a routinely used process solvent signal, for both wavelength and photometric precision, following reactor cleaning). The performance verification standard should match the format of the samples in the current analysis as closely as possible and use similar spectral acquisition parameters. Quantitative measurements of an external performance verification standard spectrum check both the wavelength (x-axis and laser wavelength) and the photometric (signal) precision. Favorable comparison of a series of performance verification spectra demonstrates proper continued operation of the instrument.

WAVELENGTH PRECISION

The wavelength precision should be measured by collecting data for a single spectrum of the selected Raman shift standard for a period equal to that used in the photometric consistency test. When appropriate, powdered samples should be repacked between each set of measurements. Peak positions across the spectral range of interest are used to calculate precision. Performance is verified by matching the current peak positions to those collected during the previous instrument qualification and should not vary with a standard deviation of more than ±0.3 cm^{-1}, although this specification can be adjusted according to the required accuracy of the measurement.

PHOTOMETRIC PRECISION

The photometric precision should be measured by collecting data for a single spectrum of a suitably verified reference standard material for a specified time. After suitable baseline correction, the areas of a number of bands across the spectral range of interest should be calculated by means of an appropriate algorithm. The area of the strongest band is set to 1, and all other envelopes are normalized to this band. Performance is verified by matching the current band areas to the respective areas collected during the previous instrument qualification. The areas should vary by no more than 10%, although this specification can be adjusted according to the required accuracy of the measurement.

LASER POWER OUTPUT PRECISION AND ACCURACY

This test is applicable only to Raman instruments with automatic, internal laser power meters. Instruments without laser power measurement should utilize a calibrated laser power meter from a reputable supplier. The laser output should be set to a representative output, dictated by the requirements of the analytical measurement and the laser power measured. The output should be measured and checked against the output measured at instrument qualification. The power (in milliwatts or watts) should vary by no more than 25% compared to the qualified level. If the power varies by more than this amount, the instrument should be serviced (as this variation might indicate, among other things, a gross misalignment of the system or the onset of failure of the laser).

For instruments with an automatic, internal laser power meter, the accuracy of the values generated from the internal power meter should be compared to a calibrated external laser power meter at an interval of not more than 12 months. The internally calculated value should be compared to that generated by the external power meter. Performance is verified by matching the current value to that generated during the previous instrument qualification. The manufacturer might provide software to

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facilitate this analysis. If the instrument design prevents the use of an external power meter, then the supplier should produce documentation to ensure the quality of the instrument and provide a recommended procedure for the above analysis to be accomplished during a scheduled service visit.

METHOD VALIDATION

Validation of Raman methods will follow the same protocols described in *Validation of Compendial Procedures* ⟨1225⟩ in terms of accuracy, precision, etc. However, several of these criteria are affected by variables specific to Raman spectrometry. Fluorescence is the primary variable that can affect the suitability of a method. The presence of fluorescent impurities in samples can be quite variable and have little effect on the acceptability of a material. The method must be flexible enough to accommodate different sampling regimes that may be necessary to minimize the effects of these impurities.

Detector linearity must be confirmed over the range of possible signal levels. Fluorescence might drive both the signal baseline and the noise higher than that used in the validation, in which case the fluorescence must be decreased, or the method modified to accommodate the higher fluorescence levels. This is also true for the precision, limit of detection, and limit of quantification of the method, as increased baseline noise will negatively impact all of these values. Because fluorescence can also affect quantification caused by baseline shifts, acceptable quantification at different levels of photobleaching, when used, should also be confirmed.

The impact of the laser on the sample must be determined. Visual inspection of the sample and qualitative inspection of the Raman spectrum for measurements with differing laser powers and exposure times will confirm that the sample is not being altered (other than by photobleaching). Specific variables to confirm in the spectrum are shifts in peak position, changes in peak height and band width, and unexpected changes in background intensity.

Method precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids, and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample holder geometry, but will vary from instrument to instrument based on excitation and collection optical configuration.

DEFINITION OF TERMS AND SYMBOLS

Calibration model: is a mathematical expression that relates the response from an analytical instrument to the properties of samples.

Instrument bandpass (or resolution): is a measure of the capability of a spectrometer to separate radiation of similar wavelengths.

Operational qualification: is the process by which it is demonstrated and documented that the instrument performs according to specifications, and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, major repair, etc.

Performance qualification: is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Qualification may employ the same or different standards for different performance characteristics.

Raman spectra:[4] are plots of the radiant energy, or number of photons, scattered by the sample through the indirect interaction between the molecular vibrations in the sample and monochromatic radiation of frequency much higher than that of the vibrations. The abscissa is usually the difference in wavenumber between the incident and scattered radiation.

(Normal) raman scattering:[4] is the inelastic scattering of radiation that occurs because of changes in the polarizability, of the relevant bonds during a molecular vibration. Normal Raman spectra are excited by radiation that is not in resonance with electronic transitions in the sample.

Raman wavenumber shift:[4]

$$\Delta\tilde{\nu}$$

is the wavenumber of the exciting line minus the wavenumber of the scattered radiation. SI unit: m^{-1}. Common unit: cm^{-1} = 100 m^{-1}.

$$\beta\Delta\tilde{\nu}$$

where β is the differential Raman cross section, is positive for Stokes scattering and negative for anti-Stokes scattering.

⟨1121⟩ NOMENCLATURE

INTRODUCTION

The *USP* (or *NF*) titles for monograph articles are legally recognized under the Federal Food, Drug, and Cosmetic Act as the designations for use in labeling the articles to which they apply.

The value of designating each drug substance by one and only one nonproprietary[1] name is important in terms of achieving simplicity and uniformity in drug nomenclature. In support of the U.S. Adopted Names program (see *Mission and Preface* in

[4] Chalmers, J., Griffiths, P., Eds. *Handbook of Vibrational Spectroscopy;* John Wiley & Sons, Ltd: New York, 2002.

[1] The term "generic" has been widely used in place of the more accurate and descriptive term "nonproprietary" with reference to drug nomenclature.

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USP–NF), of which the U.S. Pharmacopeial Convention is a co-sponsor, the USP Council of Experts gives consideration to the adoption of the U.S. Adopted Name, if any, as the official title for any compound that attains compendial recognition.

A compilation of the U.S. Adopted Names (USAN) published from the start of the USAN program in 1961, as well as other names for drugs, both current and retrospective, is provided in the *USP Dictionary of USAN and International Drug Names.* This publication serves as a book of names useful for identifying and distinguishing all kinds of names for drug substances, whether public, proprietary, chemical, or code-designated names.[2]

A nonproprietary name of a drug serves numerous and varied purposes. Its principal function is to identify the substance to which it applies by means of a designation that may be used by the professional and lay public free from the restrictions associated with registered trademarks. Teaching in the health sciences requires a common designation, especially for a drug that is available from several sources or is incorporated into a combination drug product; nonproprietary names facilitate communication among healthcare providers; nonproprietary names must be used as the titles of the articles recognized by official drug compendia; a nonproprietary name is essential to the pharmaceutical manufacturer as a means of protecting trademark rights in the brand name for the article concerned; and, finally, the manufacturer is obligated by federal law to include the established nonproprietary name in advertising and labeling.

Under the terms of the Drug Amendments of 1962 to the Federal Food, Drug, and Cosmetic Act, which became law October 10, 1962, the Secretary of Health and Human Services is authorized to designate an official name for any drug wherever deemed "necessary or desirable in the interest of usefulness and simplicity."[3]

The Commissioner of Food and Drugs and the Secretary of Health and Human Services published in the *Federal Register* regulations effective November 26, 1984, which state, in part:

> **"Sec. 299.4 Established names of drugs."**
> "(e) The Food and Drug Administration will not routinely designate official names under section 508 of the act. As a result, the established name under section 502(e) of the act will ordinarily be either the compendial name of the drug or, if there is no compendial name, the common and usual name of the drug. Interested persons, in the absence of the designation by the Food and Drug Administration of an official name, may rely on as the established name for any drug the current compendial name or the USAN adopted name listed in *USAN and the USP Dictionary of Drug Names.*"[4]

It will be noted that the monographs on the biologics, which are produced under licenses issued by the Secretary of the U.S. Department of Health and Human Services, represent a special case. Although efforts continue toward achieving uniformity, there may be a difference between the respective title required by federal law and the *USP* title. Such differences are fewer than in past revisions of the Pharmacopeia. The *USP* title, where different from the FDA Center for Biologics Evaluation and Research title, does not necessarily constitute a synonym for labeling purposes; the conditions of licensing the biologic concerned require that each such article be designated by the name appearing in the product license issued to the manufacturer. Where a *USP* title differs from the title in the federal regulations, the former has been adopted with a view to usefulness, simplicity, and conformity with the principles governing the selection of monograph titles generally.

MONOGRAPH NAMING POLICY FOR SALT DRUG SUBSTANCES IN DRUG PRODUCTS AND COMPOUNDED PREPARATIONS

The titles of *USP* monographs for drug products and compounded preparations formulated with a salt of an acid or base use the name of the active moiety, as defined below. The strength of the product or preparation is also expressed in terms of the active moiety.

An active moiety is the molecule or ion, excluding those appended portions of the molecule that cause the drug to be a salt (including a salt with hydrogen or coordination bonds), or other noncovalent derivative (such as a complex, chelate, or clathrate) of the molecule. The active moiety is responsible for the physiological or pharmacological action of the drug substance, without regard to the actual charged state of the molecule in vivo. For example, the active moiety of a hydrochloride salt of a base is the free base and not the protonated form of the base. The active moiety of a metal salt of an acid is the free acid.

This Policy is followed by USP in naming drug products and compounded preparations that are newly recognized in the *USP*. Revising existing monographs to conform to this Policy is not intended, except where the USP Council of Experts determines that, for reasons such as safety, a nomenclature change is warranted.

Labeling

The labeling clearly states the specific salt form of the active moiety that is present in the product or preparation because this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (when applicable) are provided in the labeling.

Exceptions

In rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this policy may be considered. In such cases, when the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation also is expressed in terms of the specific salt form.

[2] *USP Dictionary of USAN and International Drug Names* is obtainable on order from U.S. Pharmacopeia, Customer Service Department, 12601 Twinbrook Parkway, Rockville, MD 20852.
[3] F.D.&C. Act, Sec. 508 [358].
[4] 53 Fed. Reg. 5369 (1988) amending 21 CFR § 299.4.

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GENERAL NOMENCLATURE FORMS

List of the specific drug products with extensive examples can be found in the USP Nomenclature Guidelines (www.usp.org). USP also developed some general practices for drug product nomenclature:

- The [ROUTE OF ADMINISTRATION] is omitted for those dosage forms for which the route of administration is understood. The general form then becomes simply [DRUG] [DOSAGE FORM].
  - Thus, oral will not be included as the route of administration for orally administered capsules, tablets, and lozenges.
  - The route of administration is omitted for topically applied products—creams, ointments, lotions, and pastes. However, if some other route of administration is intended (e.g. ophthalmic), it will be included in the monograph title.
- In some instances, the drug is supplied in one dosage form for the preparation of the intended dosage form. In such cases, the dosage form provided in the container is named first and the word "for" appears, followed by the final dosage form that is suitable for administration. The general format becomes [DRUG] [DOSAGE FORM] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM]., e.g. Aspirin Effervescent Tablets for Oral Solution.
- The term "for" is included in the names of solid preparations which must be dissolved or suspended in a suitable liquid to obtain a dosage form suitable for administration, and the general format becomes [DRUG] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM]. e.g. Ampicillin for Oral Suspension, Cytarabine for Injection.
- The term "Vaginal Inserts", rather than "Vaginal Tablets", "Vaginal Capsules", or "Vaginal Suppositories" is used in the title of this type of vaginal preparation to decrease the potential for misadministration of these products.
- The term "Suppositories" is used in the titles of solid preparations that are intended for rectal administration.
- Solutions administered by injection are officially titled Injections (see *Injections and Implanted Drug Products* ⟨1⟩). The route of administration is omitted for drugs that are injected, because the route (e.g. intravenous, intramuscular, subcutaneous, etc.) must appear on the labels and in the labeling. USP defines seven types of injections:
  1. [DRUG] Injection—Liquid preparations that are drug substances or solutions thereof.
  2. [DRUG] for Injection—Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for Injections.
  3. [DRUG] Injectable Emulsion—Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.
  4. [DRUG] Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium.
  5. [DRUG] for Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Injectable Suspensions.
  6. [DRUG] Extended-Release Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium and formulated in a manner that allows the contained API to be available over an extended period of time.
  7. [DRUG] for Extended-Release Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Extended-Release Injectable Suspensions.

Injections intended to be administered through an intravenous secondary line ("piggyback") formulated in vehicles other than Water for Injection shall be named [DRUG] in [VEHICLE]. Examples of Vehicle formats which currently appear in *USP* monograph titles are:

1. [DRUG] in Dextrose Injection
2. [DRUG] in Dextrose and Sodium Chloride Injection
3. [DRUG] in Lactated Ringer's and Dextrose Injection
4. [DRUG] in Sodium Chloride Injection

POLICY FOR IMPLEMENTATION OF NOMENCLATURE REVISIONS

It is the practice of USP to set the official dates of nomenclature revisions (change of the established name or nomenclature aspects of the labeling sections in the monographs) to allow a reasonable time for product label changes to be made and to allow health practitioners and consumers time to become familiar with the new terminology. The assignment of an implementation schedule is handled by the USP Expert Committee. USP's implementation schedule, shown below, is automatic, unless an exception is sought.

18 Months

Implementation period of 18 months is usually applied when only one or a small number of products is affected.

30 Months

Implementation period of 30 months is usually applied when names or labeling of multisource products or multiproduct lines of a firm's preparations are being changed.

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60 Months

Implementation period of 60 months is usually applied for title and labeling changes that affect excipients, because such changes would require relabeling of very large numbers of prescription-only and OTC preparations.

There may be exceptions to this schedule where a shorter time is needed in order to specify nomenclature and labeling changes in cases where public health and safety are a concern. Extensions to the implementation schedule are rarely made, and must have suitable justification as well as the approval of the USP Expert Committee. Any questions or concerns regarding this postponement schedule may be addressed to the USP staff liaison assigned to the Expert Committee.

⟨1125⟩ NUCLEIC ACID-BASED TECHNIQUES—GENERAL

SCOPE

Nucleic acid-based assays are used in a variety of settings, the most common of which include the detection of infectious agents (viruses, bacteria, etc.), and cellular materials, as well as disease profiling. More recently such assays have also been used for forensic purposes and for the detection of trace contamination in biological materials. The latter include pharmaceutical development applications, such as viral clearance and adventitious agent testing in vaccine seed lots and tissue culture cell banks. This chapter introduces a series of general information chapters that provide techniques that support procedures for the detection and analysis of nucleic acids (see *Figure 1*). The assays using these techniques may be presented in a USP general chapter or in a private specification.

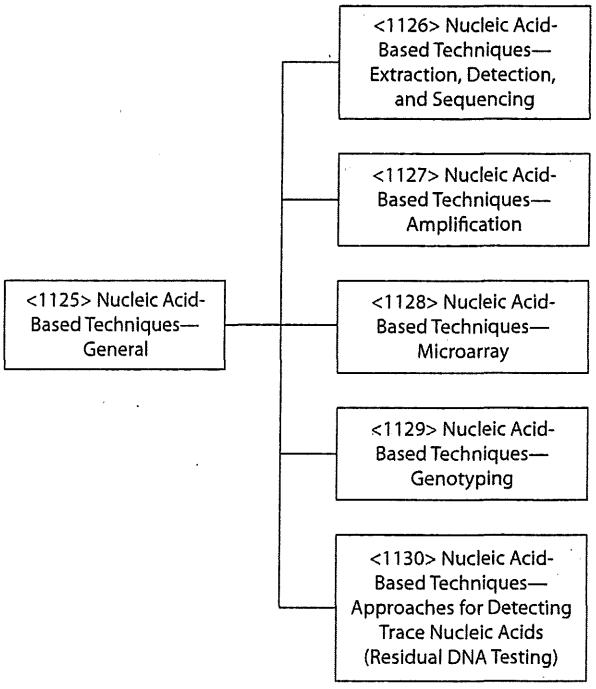

Figure 1

The major requirement for any nucleic acid analytical procedure is the availability of pure, intact nucleic acids for analysis. The information in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩ discusses procedures available for nucleic acid extraction and handling. Hybridization is the core mechanism underlying many molecular biology techniques, and in addition to the detection of nucleic acids by absorbance and fluorescence measurements and size measurement by gel electrophoresis, this chapter also covers blotting and identification of nucleic acid species by hybridization assays using labeled probes. Hybridization probes are oligonucleotides that have a sequence that is complementary to the target of interest. Probes contain radioactive, fluorescent, biotin, digoxigenin, or other tags that, upon binding of the probe to the target, allow visualization and identification of the target. Probes are capable of detecting target sequences that are present in concentrations too low to be detected by absorbance measurements or gel electrophoresis.

These analytical procedures require a minimum quantity of nucleic acid, typically in the nanogram to microgram range. However, in the vast majority of cases, e.g., in the detection of viruses or rare cellular RNA species, the nucleic acid under assay is present in minute quantities (in the picogram to femtogram range), and an amplification step must be performed before the nucleic acid can be detected and identified. The amplification step may be directed either at the signal used for detection (signal amplification), such as the branched DNA assay (bDNA assay), or at the target as in nucleic acid amplification technologies (NAT).

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In 1983 a revolutionary yet simple process termed polymerase chain reaction (PCR) was developed for amplifying the number of specific nucleic acid fragments present in a sample, and in just a few years after its discovery PCR became the most frequently used procedure for amplifying nucleic acids, especially DNA. Since the inception of PCR, the number of applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. Numerous variations of assay procedures have been developed for specific analytes. The general information chapter, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, describes amplification procedures used for DNA and RNA analysis as well as qualitative and quantitative NAT assays. Signal amplification procedures in which the signals, typically fluorescent signals, are used to detect the nucleic acid of interest, are not very common. The major signal amplification procedure, the branched DNA or bDNA assay, is used predominantly for viral nucleic acid detection.

Quality assurance aspects of the methodology are also covered, together with a summary of current regulatory requirements for NAT assays. The need for globally comparable, accurate, and reliable results in the diagnostics field has driven the quest for, and development of, national and international standards within an increasingly sophisticated and metrologically sound, highly developed international regulatory environment devoted to the highest standards of regulatory science. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT. The general information chapter, *Nucleic Acid-Based Techniques—Microarrays* ⟨1128⟩, addresses a still-emerging field that is of increasing relevance to molecular DNA analysis. Detailed treatment of various microarrays, including data analysis and validation, are excluded from ⟨1128⟩ at this time. The general information chapter, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences and common genetic variations, e.g., single nucleotide polymorphisms (SNPs). The final general information chapter in the series, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩, describes residual DNA testing in the context of pharmaceutical manufacturing. Applications relevant to viral adventitious agents, however, are discussed in the general information chapter *Virology Test Methods* ⟨1237⟩.

Two major uses of nucleic acid testing are excluded from this family of NAT chapters: viral testing for blood and blood product safety and genetic testing. The traditional perspective of USP is to develop public standards that can be applied to a particular final product without expressively defining a product and/or its production details. This chapter aims to specify when traditional methodologies or existing standards can be adapted. Novel methodologies for amplification and detection by NAT are also highlighted. As these new methodologies become mature and properly validated, they will be included in subsequent revisions.

Due to rapid development in the field, compendial and regulatory affairs scientists are advised to consult the current edition of *USP* and its *Supplements* regularly.

GLOSSARY

3′–5′ Exonuclease activity: Enzymatic activity to remove a mispaired nucleotide from the 3′ end of the growing strand. The reaction is a hydrolysis of a phosphoester bond. The presence of a 3′–5′ exonuclease, or proofreading, activity improves the fidelity of the polymerization.

5′–3′ Exonuclease activity: Enzymatic activity to remove a mispaired nucleotide from the 5′ end of a polynucleotide strand. This activity is actually that of a single-strand-dependent endonuclease and is needed to remove RNA primers of Okazaki fragments, the RNA strand in the intermediate DNA–RNA heteroduplex during reverse transcription, and during DNA repair.

Absorbance: [Symbol: A]The logarithm, to the base 10, of the reciprocal of the transmittance (T). [NOTE—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

Accuracy: The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value.

Allele: One of two or more alternative forms of a gene at a given position (locus) on a chromosome, caused by a difference in the sequence of DNA.

Amplicon: A short segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers. Sometimes referred to as an amplimer.

Amplification: The enzymatic in vitro replication of a target nucleic acid.

Annealing: Hybridizing or binding of complementary nucleic acids, usually at an optimal temperature.

Concatenation: The process in which a DNA segment composed of repeated sequences is linked end-to-end.

Complementary dna (cdna): DNA synthesized from an RNA template in an enzymatic reaction catalyzed by the enzyme reverse transcriptase.

Denaturation: The process of separating double-stranded DNA into single strands by breaking the hydrogen bonds. This is typically accomplished by heating the DNA solution to temperatures greater than 90° or by treating it with strong alkali.

Deoxyribonucleic acid (dna): The genetic material that is passed from parent to daughter cells and propagates the characteristics of the species in the form of genes it contains and the proteins for which it codes. DNA contains the following four deoxyribonucleosides: dA, dC, dT, and dG.

Deoxyribonucleotide triphosphate (dntp): A base that is added to a primer during the PCR that comprises the newly synthesized strand. Examples of dNTPs are dATP, dUTP, dCTP, dGTP, and dTTP.

Detection limit: It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

DNA polymerase: An enzyme that can synthesize new complementary DNA strands using a DNA template and primer. Several of these enzymes are commercially available, including *Taq* DNA polymerase and rTth DNA polymerase.

Endonuclease: An enzyme that cleaves phosphodiester bonds in a polynucleotide chain.

Energy transfer: This describes the process in which an excited state of one molecular entity (the donor) is deactivated to a lower-lying state by transferring energy to a second molecular entity (the acceptor), which is thereby raised to a higher energy state.

Extension: Refers to the elongation of the DNA chain that is being synthesized using the parent DNA strand as the template for synthesis of that daughter strand. This is a natural process that occurs during DNA replication. Extension occurs during the PCR process with DNA polymerases.

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Extinction coefficient: [Symbol: ε]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles/L, of the substance and the absorption path length, in cm. [NOTE—Terms formerly used include molar absorbancy index; molar absorptivity; and molar absorption coefficient.]

Fidelity: Fidelity is a measure of the accuracy of nucleic acid replication. The polymerase enzyme used is only one of the elements that influences fidelity. Other elements include buffer conditions, thermal cycling parameters, number of cycles, efficiency of amplification, and the sequence of the DNA being copied.

Fluorophore: A functional group in a molecule that makes the molecule fluorescent by absorbing energy of a specific wavelength and re-emits the energy at another wavelength.

Fluorescence: The emission of one or more photons by a molecule or atom activated by the absorption of a quantum of electromagnetic radiation. X-rays, UV, visible light, and IR radiations may all stimulate fluorescence. For details on the spectroscopic measurement of fluorescence, see *Fluorescence Spectroscopy* ⟨853⟩.

Genome: The complete genetic complement or the complete set of instructions for reproducing an organism and carrying out its biological function in life. The DNA in our cells comprises our genome. When our cells divide, the complete genome in our cells is duplicated for transmission to each of the remaining daughter cells.

Genotype: The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e., the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

Genotyping: The process of assessing genetic variations present in an individual.

Hairpin: Antiparallel duplex structure that forms by pairing of inverted repeat sequences within a single-stranded nucleic acid. The helical section is called the stem and the unpaired base segment at the end of the structure is called the loop.

Hot-start PCR: Technique that is commonly used to improve the sensitivity and specificity of PCR amplification. A hot start is performed by withholding from the reaction mix a key component necessary for amplification until the reaction reaches a temperature above the optimal annealing temperature of the primers. The component withheld from the reaction mix can be primers, DNA polymerase, $MgCl_2$, or dNTPs.

Hybridization: The process of forming a double-stranded nucleic acid molecule, for example between a nucleotide sequence (probe) and a target.

Ligation: The process of joining two or more DNA fragments.

Melting temperature (T_m): The temperature at which 50% of the DNA becomes single-stranded.

Microarray: Sets of miniaturized chemical reaction areas that are used to test DNA fragments, antibodies, or proteins. Usually the probes are immobilized on a chip and hybridized with target.

Mismatch: Unconventional base pairing (other than C with G, and A with T or U). A mismatched base pair has lower bonding energy and decreases the stability of the DNA molecule.

Nucleic acid: Linear polymers of nucleotides, linked by 3′, 5′ phosphodiester linkages. In DNA, deoxyribonucleic acid, the sugar group is deoxyribose, and the bases consist of adenine, guanine, thymine, and cytosine. RNA, ribonucleic acid, has ribose as the sugar, and uracil replaces thymine.

Oligonucleotide: Linear sequence comprising as many as 25 nucleotides joined by phosphodiester bonds, generally used as a DNA synthesis primer.

Photobleaching: Photobleaching is the irreversible destruction of a fluorophore in the excited state. Different fluorophores have different rates of photobleaching. For example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. A simple and practical way to overcome this is to reduce the incident radiation.

Polymerase: An enzyme that catalyzes the synthesis of nucleic acids on pre-existing nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

Polymerase chain reaction (PCR): A laboratory technique that rapidly amplifies a specific region of double-stranded DNA, predetermined by the pair of primers used for amplification. Generally involves the use of a heat-stable DNA polymerase.

Precision: The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample.

Primer: Nucleic acid polymerases link a mononucleotide to a chain of nucleic acids, which is called the primer. RNA polymerases are able to use a single nucleotide as a primer, but DNA polymerases always require an oligonucleotide.

Probe: A specific DNA or RNA sequence that has been labeled by radioactive, fluorescent, or chemiluminescent tags and is used to detect complementary sequences by hybridization techniques such as blotting or colony hybridization. In addition, probes can also be used for quantitation of amplicons as described for quantitative PCR assays. A more detailed description of such probes is given in the general information chapter, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

Processivity: The ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate.

Proofreading activity: Literally to read for the purpose of detecting errors for later correction. DNA polymerase has a 3′ to 5′ exonuclease activity that is used during polymerization to remove recently added nucleotides that are incorrectly paired.

Quantitation limit: It is the lowest amount of analyte in a sample that can be determined with an acceptable precision and accuracy under the stated experimental conditions.

Quenching: The process of extinguishing, removing, or diminishing a physical property such as heat or light. Fluorescence quenching can be either collisional or static.

Reverse transcriptase: An enzyme that requires a DNA primer and catalyzes the synthesis of a DNA strand from an RNA template. An enzyme that can use RNA as a template to synthesize DNA.

Reverse transcription (rt): The process of making cDNA using an RNA template.

Real-time PCR: May often be referred to as Quantitative PCR or Real-Time Quantitative PCR but not RT-PCR and is a procedure for simultaneous DNA quantitation and amplification. The generation of amplicons monitored as they are generated by the use of a fluorescent reporter system and captured by sophisticated instrumentation.

Real-time (RT-PCR): The combination of real-time PCR and reverse transcription PCR.

Reverse transcriptase polymerase chain reaction (RT-PCR): A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

Ribonucleic acid (RNA): A type of nucleic acid composed of a specific sequence of ribonucleotides linked together. RNA contains the following four ribonucleosides: A, C, G, and U.

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Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage.

rTth dna polymerase: Recombinant thermostable DNA polymerase originally isolated from the bacterium *Thermus thermophilus*. rTth has optimal activity at 70°–80° and survives the denaturation steps of PCR. In addition to DNA polymerase activity, it has efficient reverse transcriptase activity in the presence of manganese.

Specificity: The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation proeducts, and matrix components.

Taq dna: Thermostable DNA polymerase that is originally isolated from the bacterium *Thermus aquaticus*, *Taq* has optimal activity at 70°–80° and is not degraded during the high-heat denaturation steps of PCR.

Template: A master copy used to start the DNA or RNA replication process.

Transcription: The synthesis of RNA using a DNA template.

APPENDICES

Appendix 1: Regulations and Standards

Nucleic acid-based techniques have rapidly transformed almost every field of research, pharmaceutical development, and diagnostics. The need for globally comparable, accurate, and reliable results in the diagnostic field has driven the development of national and international standards as well as fostered a highly developed regulatory environment. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT.[1] Virus-specific regulations and reference standards will be addressed in the Appendix to General Information chapter *Virology Test Methods* ⟨1237⟩. The following is a selective list of national guidance documents. For application-specific guidance the compendial user is referred back to the relevant regulatory agency for the most current guidance.

- FDA Center for Biologics Evaluation (CBER) "Nucleic Acid Based In Vitro Diagnostic Devices for Detection of Microbial Pathogens" (2005)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Biological In Vitro Diagnostic Product" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: In the Manufacture and Clinical Evaluation of In Vitro Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Viruses Types 1 and 2" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Use of Nucleic Acid Tests on Pooled and Individual Samples from Donations of Whole Blood and Blood Components (including Source Plasma and Source Leukocytes) to Adequately and Appropriately Reduce the Risk of Transmission of HIV-1 and HCV" (2004)

Appendix 2: Abbreviations

AABB	American Association of Blood Banks
ACD	acid citrate dextrose
ASO	allele-specific oligonucleotides
bDNA	branched DNA assay
BMA	bone marrow aspirate
CE-LIF	capillary electrophoresis and laser-induced fluorescence
CCD	charge-coupled device
cDNA	complementary DNA
CPR	cyclic probe reaction
CsCl	cesium chloride
Ct	cycle threshold
DEPC	diethylpyrocarbonate
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dNTP	dinucleotide triphosphate
DOP-PCR	degenerated oligonucleotide primed PCR

[1] Reference materials for nucleic acid-based techniques are available from National Institute of Standards and Technology (NIST), http://ts.nist.gov/measurementservices/referencematerials/index.cfm.

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dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
dUTP	2′-deoxyuridine 5′-triphosphate
dTTP	2′-deoxythymidine 5′-triphosphate
ESI	electrospray ionization
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FEN	flap endonuclease
FISH	fluorescent in situ hybridization
FFPE	formalin-fixed paraffin embedded
FRET	fluorescence resonance energy transfer
GLP	good laboratory practice
HCV	hepatitis C virus
HIV	human immunodeficiency virus
ICH	International Conference on Harmonization
LAPS	light-addressable potentiometric sensor
LCR	ligase chain reaction
LED	light-emitting diode
LNA	locked nucleic acid
MALDI	matrix-assisted laser desorption–ionization
MDA	multiple-displacement amplification
MOPS	3-[*N*-morpholino]propanesulfonic acid
MS	mass spectrometry
mRNA	messenger RNA
NAT	nucleic acid amplification technologies
NASBA	nucleic acid sequence-based amplification
NTP	nucleotide triphosphate
OLA	oligonucleotide ligation assay
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	primer-extension-preamplification
PPi	pyrophosphate
QA	quality assurance
QC	quality control
RCA	rolling circle amplification
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
rTth	recombinant *Thermus thermophilus*
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
3SR	self-sustained sequence replication
SSCP	single-strand conformation polymorphism
STR	short tandem repeat
Taq	*Thermus aquaticus*

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Tm	melting temperature; the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded
TMA	transcription-mediated amplification
TOF	time-of-flight
UNG	uracil-*N*-glycosylase
WGA	whole-genome amplification

⟨1126⟩ NUCLEIC ACID-BASED TECHNIQUES—EXTRACTION, DETECTION, AND SEQUENCING

NUCLEIC ACID EXTRACTION

Introduction

The basic principles of nucleic acid amplification technology (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. The current chapter covers general steps in the extraction and purification of nucleic acids from a variety of samples.

The expanding discipline of molecular biology in pharmaceutical and biomedical research and development is characterized by the rapid discovery of new markers for disease and technologies for their detection. Nucleic acid targets are isolated from a wide variety of specimens, and the quality and quantity of the extracted target are highly affected by specimen collection, handling, and choice of extraction procedure.

The analysis of complex organisms by molecular biological techniques requires the isolation of pure, high molecular weight genomic DNA and intact full-length RNA. The application of these techniques then allows the detection, identification, and characterization of the associated organism or adventitious agent. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay–Sachs disease, to name a few examples, or the analysis of single nucleotide polymorphisms (SNPs).

DNase and RNase are the major sources of nucleic acid instability. Although both enzymes are ubiquitous and are easily released during nucleic acid extraction, RNases are far more stable and harder to inactivate than are DNases because they generally do not require co-factors in order to function. Minute amounts of RNase are sufficient to destroy RNA, so great care should be taken to avoid inadvertently introducing these enzymes into the sample during or after the isolation procedure. If RNA is collected for the specific application of gene expression analysis, researchers should keep in mind that the sample collection process itself can alter the resulting expression profile.

Because of the ubiquity of RNases, measurement of intracellular RNA targets has lagged behind that of DNA targets in contributing to patient management and characterization of targets for pharmaceutical purposes. However, RNA represents the current status of the organism and is an important tool for correlating a phenotype with its associated genetic activity. The unstable nature of RNA has made standardization of NAT tests difficult, and false negative results can easily arise from a poorly handled sample because of target degradation rather than from the absence of disease or regulation of gene activity. Nevertheless, commercially available isolation and detection systems provide a high level of standardization and robustness, resulting in the implementation of RNA-based assays in recent years. The following sections discuss general steps in the extraction and purification of nucleic acids from a variety of samples, focusing on (1) collection, handling and storage of samples; (2) disruption of samples; (3) subsequent extraction and purification of nucleic acids; and (4) storage of purified nucleic acids.

Sample Source

The broad diversity of possible specimens requires different procedures for collection. For example, blood samples are collected in an appropriate anticoagulant- or additive-containing tube. Ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests that require plasma or bone marrow aspirate (BMA) samples. When extraction from tissues is appropriate, the optimal amount of tissue is usually 1 to 2 g, depending on the type of tissue, because the amount of DNA and RNA per weight of tissue varies greatly from tissue to tissue. In general, more than 10 mg of tissue is required to obtain >10 µg of DNA or RNA. Because of the highly variable amounts and types of proteins and other contaminants present in different tissues, nucleic acid isolation protocols are tissue-specific, and a broad range of ready-to-use isolation systems are available from different manufacturers of kits for nucleic acid extraction. The tissue type also influences the stability of both DNA and RNA in specimens, and the two types of nucleic acid differ importantly with respect to sample preparation and downstream analysis. These issues are described later in the chapter.

Pre-Analytical Steps and Sample Collection

Although the genetic makeup of the organism remains mostly unchanged over time, the mRNA population represents the current status of a cell under any given set of conditions, and thus is highly dynamic. To prevent degradation of mRNA and/or to preserve the original transcription pattern of the cellular mRNA, tissue should be placed immediately on ice or snap-frozen in liquid nitrogen. However, freezing disrupts the cellular structure and releases RNases. Hence, for RNA isolation in general (mRNA, ribosomal RNA, viral RNA, etc.), thawing in an RNase-inactivating buffer is essential. A more convenient procedure

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employs a stabilizing agent at ambient temperature. Several reagents for different types of sample material (e.g., tissue or bacteria) are commercially available. Vanadium salts were once used to inhibit RNase activity, but they have been superseded by the use of chaotropic agents for the inhibition of RNase and stabilization of RNA. The sample can easily be collected in such reagents and stored for several days to weeks prior to RNA isolation.

For reliable gene-expression analysis, the immediate stabilization of the RNA expression pattern and of the RNA itself is an absolute prerequisite. Directly after the biological sample is harvested or extracted, changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as transcriptional induction. Such changes in the gene-expression pattern should be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

The use of gloves while handling reagents and RNA samples is mandatory to prevent RNase contamination arising from contact with the surface of the skin or from laboratory equipment. In order to create and maintain an RNase-free environment, laboratory personnel should treat water or buffer solutions with diethylpyrocarbonate (DEPC), which inactivates RNases by covalent chemical modification. Care should be taken because DEPC is irritating to the eyes, skin, and mucous membranes and is also a suspected carcinogen. Alternatively, commercially available RNase-free solutions and reagents may be used. Commercially available RNase inhibitor proteins are also available for use in reactions but with different levels of effectiveness with respect to various RNase types. However, it should be noted that DEPC cannot be used with Tris-buffered solutions. Many scientists recommend the use of disposable vessels when working with RNA. Nondisposable glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240° for 4 or more hours before use (autoclaving alone will not fully inactivate many RNases). Alternatively, glassware can also be treated with DEPC. Nondisposable plasticware should be thoroughly rinsed with 0.1 M sodium hydroxide and 1 mM EDTA, followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. The use of aerosol-resistant filter tips is also important for avoiding RNase contamination. These issues are not critical for DNA, and following the rules of Good Laboratory Practice (GLP) is generally sufficient for successful isolation of DNA.

As a general precaution, staff should follow all applicable safety precautions when handling tissue or body fluids (human or other). Some of these precautions (e.g., the use of disposable gloves) also prevent contamination of the sample. Applicable guidelines and standards for the collection and processing of human-derived materials have been published by the American Association of Blood Banks, the International Conference on Harmonization, and the FDA.

Sample Disruption and Homogenization

Prior to extraction, source material is disrupted and homogenized. Disruption is the complete breakage of cell walls and plasma membranes of solid tissues and cells in order to release all DNA and RNA contained in the specimen. This is usually done using a lysis buffer that also inactivates endogenous nucleases. In addition to disrupting tissues, homogenization shears high molecular weight DNA and cellular components. During RNA isolation, scientists often must reduce the viscosity of cell lysates (caused by the presence of high molecular weight DNA molecules) prior to final isolation in order to make the subsequent extraction steps easier and more efficient. Incomplete homogenization may interfere with subsequent RNA purification steps (e.g., inefficient binding of RNA to silica membranes) and therefore result in significantly reduced yields. A typical procedure to shear high molecular weight DNA and homogenize the sample is to repeatedly pass the lysate through a small-gauge needle. However, this procedure is time-consuming and is not suitable for high throughput of samples. Better procedures to achieve complete disruption and homogenization of cells and tissue include rapid agitation in the presence of beads and lysis buffer (bead milling) or rotor–stator homogenization.

During the bead milling process, disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the size and composition of the beads, the speed and configuration of the agitator, the ratio of buffer to beads, the disintegration time, and the amount of starting material. These parameters must be determined empirically for each application. For disruption with mortar and pestle, the samples should be frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. Standard safety precautions and the use of safety clothing to protect the skin and eyes should be employed when working with liquid nitrogen. Rotor–stator homogenizers are able to disrupt and homogenize animal and plant tissues within 5 to 90 seconds, depending on the sample. The rotor turns at very high speed, causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Other alternatives are commercial spin-column homogenizers in combination with silica-membrane technology, which provide a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples.

In order to achieve complete disruption, different sample types require different procedures. Cells from tissue culture grown as a monolayer or in suspension are easily disrupted by the addition of a lysis buffer that typically contains a mixture of an anionic detergent, a protease, and a chaotropic agent in a buffered salt solution. In contrast, nucleic acid isolation from fibrous tissues such as skeletal muscle, heart, and aorta can be difficult to disrupt because of the abundance of contractile proteins, connective tissue, and collagen. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using a lysis buffer and a proteinase.

In general, the same procedures are applicable for extraction of DNA and RNA. For DNA isolation more gentle procedures are preferable, but during RNA isolation, cells and tissues can be disrupted using a mixer mill because there is no risk of shearing the RNA. Certain downstream applications require high molecular weight DNA, and care should be taken not to shear the DNA molecules and thus render the DNA unsuitable for further analysis.

Extraction and Purification

Although several procedures are available for nucleic acid extraction, the suitability of a procedure depends on the starting material, the type and purity of nucleic acid isolated, and possibly the downstream application. The principal procedures are described below; several commercial kits are available to accommodate different sample types and applications.

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PHASE EXTRACTION

The original technique for extraction of DNA and RNA from lysed samples is phase extraction, which involves nucleic acid extraction using a mixture of phenol and chloroform. Depending on pH and salt concentration, either DNA or RNA partitions in the aqueous phase. At neutral/basic pH, the DNA remains in the aqueous phase, and RNA remains in the organic phase or in the interphase (with the proteins). However, at acidic pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA, which remains charged, partitions in the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, followed by extraction with chloroform to remove any residual phenol. The nucleic acid is recovered from the aqueous phase by precipitation with alcohol. For RNA, this procedure is often combined with a protease digestion, alcohol or lithium chloride precipitation, and/or cesium chloride (CsCl) density gradients. A potential problem is contamination of the recovered DNA or RNA with organic solvents that may interfere with enzymatic downstream applications or spectrometry readouts.

CESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

For the isolation of high molecular weight genomic DNA, CsCl density gradient centrifugation is the traditional procedure. Cells are lysed using a detergent, and the DNA is isolated from the lysate by alcohol precipitation. The DNA is then mixed with CsCl and ethidium bromide and centrifuged for several hours at a high g force (typically 100,000 × g). The DNA band, which can be visualized under UV light as a result of the intercalation of the ethidium bromide with the DNA, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA. This procedure allows the isolation of high-quality DNA, but it is time consuming and also a safety concern because of the high quantity of EtBr involved.

ANION-EXCHANGE CHROMATOGRAPHY

An alternative procedure for the purification of high molecular weight genomic DNA is anion-exchange chromatography based on the interaction between the negatively charged phosphate groups of the nucleic acid and positively charged surface molecules on the anion-exchange resin. Binding occurs under low-salt conditions, and impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers. Pure DNA is eluted with a high-salt buffer and is desalted and concentrated by alcohol precipitation. This procedure yields DNA of a purity and biological activity equivalent to two rounds of purification in CsCl gradients, but in much less time. The procedure also avoids the use of toxic substances, and it can be adapted for different scales of purification. DNA up to 150 kilobases (kb) in length may be isolated using this procedure. Several kits are available for the isolation of DNA based on anion-exchange technology, and procedures vary in processing times and the quality and size of the isolated DNA.

SILICA TECHNOLOGY

The current procedure of choice for most applications is based on silica technology and can be used for isolation of full-length RNA or DNA with an average size of 20 to 50 kb. However, higher molecular weight DNA exceeding 100 kb is not efficiently extracted by this technology. The procedure relies on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts. Although both types of nucleic acid adsorb to silica, the use of specific buffers in the lysis procedure ensures that only the desired nucleic acid is adsorbed while other nucleic acids, cellular proteins, and metabolites remain in solution. The contaminants are washed away, and high-quality RNA or DNA is eluted from the silica using a low-salt buffer. The silica matrix can be used as particles in suspension, in the form of magnetic beads, or as a membrane. This technique is suitable for high throughput, and several kits and automated systems are commercially available. However, these aqueous lysis buffers (in contrast to lysis buffers based on an organic solvent such as phenol) are not ideally suited for difficult-to-lyse samples (e.g., fatty tissues). Kits designed to facilitate lysis of fatty tissues and to inhibit RNases are available. Silica-based kits provide a fast and reliable procedure for both DNA and RNA purification and are commonly used for nucleic acid extraction.

Although these procedures yield pure nucleic acids, for some applications in which even trace contaminations with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. Alternatively, procedures that use specific probe capture may be used. Relevant applications requiring such ultra-pure nucleic acids are discussed in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

Specific Applications for Hard-to-Extract Materials

EXTRACTION FROM FORMALIN-FIXED AND PARAFFIN-EMBEDDED BIOPSIES

The nucleic acids in formalin-fixed paraffin embedded (FFPE) biopsies are usually heavily fragmented and chemically modified by formaldehyde. Although formaldehyde modification cannot be detected in standard quality control assays such as gel electrophoresis, formaldehyde modification does interfere with enzymatic analyses. Sufficient extraction and demodification for DNA can be achieved by prolonged digestion with protease, but this will lead to heavy fragmentation and degradation of RNA. Some isolation systems have been optimized to reverse as much formaldehyde modification as possible without further RNA degradation. Nevertheless, RNA purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR).

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EXTRACTION FROM BACTERIA AND PATHOGENS

Although Gram-negative bacteria are relatively easy to lyse, Gram-positive bacteria or yeasts typically need an enzymatic pretreatment to remove the cell wall for efficient lysis. This methodology can be applied only to DNA isolation because the enzymatic treatment will influence the expression profile of the organism, and therefore RNA isolation requires a more rapid lysis procedure. Another factor to consider is that microorganisms normally occur against the background of a host or an environmental matrix (e.g., soil), which makes detection by polymerase chain reaction (PCR) often difficult because of inhibitory components. This means that the isolation procedure has to be carefully adapted and optimized for the specific organism and sample type. Commercial kits are available, and most are based on the use of lysozyme for the removal of cell walls.

SPECIAL CONSIDERATIONS FOR LIMITED SAMPLE AMOUNTS

Multiple genetic testing techniques, including SNP analysis, short tandem repeat analysis, sequencing or genotyping using arrays, real-time PCR, and other procedures depend on the availability of high-quality DNA. Because human genomic DNA or samples of individual genotypes are often limited, a process to immortalize nucleic acid samples can overcome this limitation. Procedures applicable to genotyping are discussed in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩. Whole-genome amplification (WGA) has recently been employed to amplify limited genomic DNA from already purified DNA or directly from clinical or casework samples without any DNA purification. Two basic technologies for WGA are available and are PCR-based or rely on isothermal multiple-displacement amplification. These applications are described in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

Sample Handling and Long-Term Storage

DNA is a relatively stable macromolecule, and once isolated it can be kept at 2° to 8° for at least 1 year. However, where DNA is present in very small quantities, such as in a test of residual DNA, it may be advisable to store the DNA at less than or equal to –20°. Generally, DNA is stored in solution. Distilled water can be used if DNA will be used for PCR and/or endonuclease digestion within a few days after its isolation. However, Tris–EDTA at pH 7.5–8.5 is the preferred buffer for DNA storage because DNA degradation can occur in water because of the limited buffering capacity of this medium. Purified nucleic acids retain recognizable characteristics during long-term storage, provided the samples are stored as frozen solutions. The DNA solution should be stored as a primary stock solution frozen at –80°. DNA can also be lyophilized and stored dry without the need for refrigeration. In some cases DNA can be stored for years on special filter papers that bind DNA and allow storage in a dried state at ambient temperature.

The ubiquity of RNases requires extra precautions when handling RNA. Isolated RNA should be kept on ice when aliquots are pipetted. Filter tips that prevent RNase carry-over from the pipette and sterile, disposable polypropylene tubes are recommended throughout the procedure because these tubes are generally RNase-free and do not require any pretreatment to inactivate RNases. Purified RNA can be stored at –20° or –80° in water. Under these conditions no degradation is normally detectable. Unlike DNA, RNA does not benefit from basic buffer solutions during long-term storage because of its sensitivity to alkaline conditions. Generally, if nucleic acid samples are required for multiple testing, RNA and DNA samples should be frozen in multiple aliquots at –80° for subsequent analysis in order to avoid repeated freeze–thaw cycles that can lead to degradation, and also to minimize the possibility of contamination, which could result in analytical inaccuracy.

QUALITATIVE AND QUANTITATIVE EVALUATION OF NUCLEIC ACIDS

Introduction

This section describes procedures that assess the purity, integrity, and quantity of purified nucleic acids, including spectroscopic procedures, electrophoresis of nucleic acid fragments, and probe-based techniques. Detection and quantitation by amplification are discussed in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

ABSORBANCE SPECTROSCOPY

The basic principles of spectroscopy are addressed in *Ultraviolet-Visible Spectroscopy* ⟨857⟩. For nucleic acids, absorbance is determined at 260 nm, but this procedure does not distinguish between DNA and RNA. Absorbance can also be used to estimate protein contamination in nucleic acids. Proteins maximally absorb at 280 nm, and nucleic acids maximally absorb at 260 nm. Thus the calculation of the A260/A280 ratio is used as an estimation of protein contamination in nucleic acid preparations. A ratio of 1.8 to 2.0 is considered desirable. As an example, double-stranded DNA has an extinction coefficient of 20 for 1 mg per mL of DNA at 260 nm and a coefficient of 10 at 280 nm. In contrast, for 1 mg per mL of protein, the extinction coefficients are on the order of 1 at 280 nm (depending on tyrosine and tryptophan content) and 0.57 at 260 nm. Thus a large protein contamination could exist at a 260/280 ratio of greater than 1.8 because of the lower sensitivity of protein absorbance. In addition, the change of absorbance of DNA with wavelength ($\Delta A/\Delta\lambda$) is steep at 280 nm, and this could lead to an incorrect determination if the spectrophotometer is out of calibration. The peak at 260 nm is broad, and thus readings are less sensitive to calibration issues.

Information on contamination by nonproteinaceous materials can be provided by a scan of DNA from 220 nm to 320 nm. Pure DNA has a mostly symmetric peak around 260 nm, zero absorbance at 320 nm, and a minimum at 230 nm. Absorbance rises again from 230 nm to 220 nm. Interfering substances can co-purify with DNA and absorb in the lower UV range (around 230 nm). These substances can interfere with and lead to an overestimation of DNA content, thus showing the utility of a scan —or at least a measurement of absorbance—at 230 nm in addition to 260 nm and 280 nm. Absorbance above 300 nm can arise from other contaminants and particulate matter. Common reagents used in the isolation of DNA, particularly solvents

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such as phenol and alcohols if they are not completely removed, can interfere with DNA absorbance measurements. Analysts should be aware of the limitations of this type of measurement. Finally, the absorbance of DNA and the 260/280 ratio is dependent on ionic strength—a difference as large as 30% can exist. Absorbance of genomic DNA is higher, and the 260/280 ratio is lower in pure water when compared with the same DNA in a buffer or a salt solution.

For the purposes of quantitation of nucleic acids, the respective extinction coefficients for DNA and RNA are used. An absorbance of 1 in a 1-cm cuvette corresponds to 50 µg per mL of double-stranded DNA [E (specific absorption coefficient) = 0.02 (µg per mL)$^{-1}$ cm^{-1}]. The specific absorption coefficient for RNA at 260 nm is E = 0.025 (µg per mL)$^{-1}$ cm^{-1} (absorbance of 1.0 corresponds to 40 µg per mL), and for single-stranded DNA E = 0.027 (absorbance of 1.0 corresponds to 37 µg per mL). A solution of DNA is read against a blank of the same buffer solution in which the DNA is dissolved. Ideally, readings should fall within a range of 0.1 to 1.0 absorbance for adequate linearity. Absorbance above 1.0 becomes increasingly nonlinear as the absorbance rises. The accuracy of readings below 0.1 (5 µg per mL DNA) depends on the quality and noise level of the spectrophotometer.

Fluorescence Protocols for DNA and RNA Quantitation

Cyanine dye derivatives are used for the quantitation of nucleic acids because they specifically interact with nucleic acids (DNA, RNA, and oligonucleotides) and fluoresce only upon binding. The exact mechanism of interaction is not always fully understood but may involve intercalation in double-stranded DNA and surface binding.

Measurements can be performed using a fluorometer or a plate reader. The sensitivity of fluorescence with these dyes is much higher than that of absorbance, which gives these dyes great utility when DNA concentration is low (down to 25 pg per mL). The dye must be protected from light to avoid photobleaching. Linearity is maintained over three to four orders of magnitude. Calf thymus and Lambda phage DNA are often used as calibrants to construct a standard curve. Some of these dyes have been optimized to bind double-stranded DNA or single-stranded RNA and oligonucleotides. A DNA-binding dye will also bind to single-stranded DNA and RNA but at low ionic strength, and the signal is about 10% or less than that seen with double-stranded DNA for an equivalent mass of material. Thus, this methodology is preferred for DNA measurements when no effort has been made to remove RNA from the preparation. Another fluorescent dye is available and is optimized for RNA measurements. Using two different concentrations of this dye, analysts can detect RNA in amounts as low as 1 ng per mL and as high as 1 µg per mL. The dye also fluoresces with DNA but does not display an equivalent ability to minimize binding by the use of particular conditions (e.g., with DNA and the double-strand binding dye). Quantitation may be affected by contaminating nucleic acid (e.g., DNA in an RNA preparation and vice versa). Treatment with a DNase is needed if DNA is present in the RNA preparation. Proteins are unlikely to interfere with these dyes, but some detergents as well as phenol result in loss of fluorescence. Nucleic acid extraction reagents should thus be checked for effect on subsequent fluorescent assays.

Bisbenzimide fluorochrome dyes such as (2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1*H*-benzimidazole) represent another option for measuring DNA. Researchers have studied the binding of these dyes to the minor groove of DNA and have found that sequences of adenine or thymine in the DNA sequence provide a minor groove dimension that binds the dyes best. Thus the fluorescent signal can show DNA sequence dependence, and the calibrant DNA should have a nucleotide composition that is similar to that of the DNA to be measured. These dyes are not as sensitive as cyanine dyes but are more sensitive than absorbance measurements. Low dye concentrations and high ionic strength are required in order for analysts to distinguish double-stranded DNA from RNA. Low ionic strength conditions are required in order to differentiate double-stranded DNA from single-stranded DNA.

Detection by Size

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis provides a simple and accurate procedure for separating nucleic acids by fragment size. The technique can be adapted to separate fragments over a large range of sizes and can be used in a preparative or analytical fashion. For example, gel electrophoresis can be used to verify that a product of a PCR reaction is of the correct size. DNA fragments can be retrieved from a gel slice and provide a sufficiently pure PCR product for cloning or sequencing. The general integrity of an RNA preparation can be determined by gel electrophoresis as well. The stoichiometry of the nucleic acid fragment size (in base pairs) and negative charge from the phosphate provide the basis for the separation. With the exception of plasmids, electrophoresis is generally free of DNA conformation-induced effects. Supercoiled plasmid DNA will migrate ahead of linear or open-circle/nicked plasmid, which is useful for determining the conformation of a plasmid preparation. In contrast, denaturing gels are used for RNA because of RNA's tendency to form inter- and intramolecular secondary structures.

Agarose gel electrophoresis utilizes a horizontal setup wherein the gel is cast in a box and placed on a bridge between two buffer compartments that are filled with the buffer of choice. The gel is also covered with a thin layer (~1 mm) of buffer. Although the main electrical resistance resides in the gel itself, there is sufficient charge on the nucleic acids to move fragments through the gel toward the anode. The fragments move in proportion to size, the smallest moving the fastest. The parameters that most affect electrophoresis are gel pore size, buffer concentration, and the voltage gradient. The ability to separate the fragments of choice is largely a function of the gel pore size, which depends on agarose concentration. Generally the agarose concentration is in the range of 0.5% to 1.0% for DNA fragments of <100 to 25,000 base pairs, and the higher concentration is used when it is important to distinguish the smallest fragments. Lowering the agarose concentration in the gel results in the resolution of larger fragments but also in a loss of resolution of small fragments. For the largest fragments pulsed, (reversed)-field electrophoresis is utilized.

To achieve uniform electrophoresis, all of the agarose must be completely melted. Electrophoresis-grade agarose is dissolved in the same buffer that will be used for electrophoresis. The buffers most commonly used for DNA separations are TBE (tris-borate-EDTA) or TAE (tris-acetate-EDTA). TBE has a higher buffering capacity than TAE, but TAE should be used if the DNA is going to be retrieved from the gel. Denaturing RNA gels use MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM

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EDTA, pH 7.0). Melting the agarose is conveniently achieved with the assistance of a microwave oven. The agarose will easily come to a boil, but this may not result in complete melting of the agarose, which may require bringing the solution to a boil several times, with intermittent mixing and holding periods, until the agarose is completely melted. Agarose particles transform from white to transparent before melting. Any partially melted agarose can be detected by swirling the flask while holding it up to the light. If the solution does not appear uniform, then it requires additional heating. The agarose is poured into the gel box after partial cooling but before setting up. Commercially available ready-to-use gels suitable for a particular application can also be used. For RNA-denaturing gels, formaldehyde is added under a fume hood to the melted agarose to a final concentration of 2.2 M or 6.7%. Before the agarose has hardened, the analyst places a comb in the gel to provide wells for the samples and size standards. Once solidified, the gel is placed in the electrophoresis box, and buffer is added until both sides are filled and there is a layer of buffer across the surface of the gel. Then 10X tracking buffer (40% sucrose with 0.25% bromphenol blue or 0.25% xylene cyanol or both) is added to each DNA sample to increase the sample density and to provide a tracking dye that is used to assess when the electrophoresis is finished. The increased density allows the sample to be transferred into the well and to remain there until it migrates into the gel during electrophoresis.

One or more lanes should be used for a DNA size standard containing fragments in the range that is relevant to the samples and agarose concentration. Size standards in various ranges are readily available. Bracketing the samples in wells between standards is useful to determine whether the electrophoresis gradient has been uniform over the width of the gel. However, in the case of eukaryotic RNA preparations, the 18S and 28S ribosomal RNAs that are co-extracted from prominent bands (corresponding to 1900 and 4700 nucleotides) can also be used as size standards. In addition, the rRNA provides information on the RNA integrity because missing or fuzzy rRNA bands indicate problems with the quality of the RNA preparation. Once the wells are filled, the cover is placed over the gel box, and the box is connected to the power supply. The indicator dye in the tracking buffer added to the samples and size standard allows the easy determination of how far the electrophoresis has proceeded. Bromophenol blue will migrate with DNA fragments of <500 base pairs, and xylene cyanol will migrate with fragments of 5000 base pairs.

The power supply is frequently run under conditions of constant voltage (1 to 10 V per cm) of gel length. Elevated voltage can cause high current, resulting in the generation of damaging heat and exhaustion of the buffer.

PULSED-FIELD ELECTROPHORESIS

This variation on agarose gel electrophoresis is used to separate a range of large DNA fragments and is most useful when resolution of 50,000 to 200,000 base-pair fragments is needed. The main difference is the addition of an alternating-field device that controls the power supply operating under constant voltage. Large fragments of DNA change conformation in order to move through the agarose pores, and the larger pieces take longer to readjust when the field is reversed and thus move more slowly than do smaller fragments. This allows resolution of fragments over the period of hours that the pulsed-field procedure operates. A commonly used ratio of forward to reverse is 3:1, and, in addition, the procedure typically calls for a stepwise increase in the unit time between reverses of the field. Electrophoresis may continue for 10 to 16 hours to avoid fluctuation in gel temperature, viscosity, and other properties that may cause artifacts.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The format for performing PAGE is quite different from that for agarose gel electrophoresis. The general procedure for PAGE is described in *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩. For resolution of small fragments of DNA in the 10 to 500 base-pair range, nondenaturing polyacrylamide gel electrophoresis is more suitable than agarose gel electrophoresis because separation of fragments of this size requires much smaller pore size than is achievable in agarose gels. The gel is prepared by polymerization of acrylamide monomers. The percentage of acrylamide dictates the range of fragment sizes that can be best resolved. For example, 20% acrylamide is suitable for the 10–100 base-pair range, and 5% acrylamide is useful in the 100–500 base-pair range. Commercially available ready-to-use polyacrylamide gels suitable for the particular size discrimination can also be used. The separated nucleic acids are visualized by staining with, for example, silver nitrate solution rather than with ethidium bromide or cyanine dye. However, staining with silver nitrate is laborious and time-consuming and not suitable for preparations that contain a large amount of protein, because proteins will also stain with silver nitrate.

CAPILLARY ELECTROPHORESIS AND LASER-INDUCED FLUORESCENCE (CE–LIF)

CEF has been used for many years to separate DNA fragments (for the general principles of CE, see *Capillary Electrophoresis* ⟨1053⟩). The procedure relies on a principle similar to that underlying agarose gel electrophoresis. CE can utilize the cross-linked buffer systems applied in gel electrophoresis, but the technique can also use polymer-containing solutions (e.g., polymethylcelluloses) that are designed to create pores that entangle proteins. These polymer solutions may be added to the capillary between injections, allowing a "fresh" gel prior to each run. In addition, capillaries can be used for more injections than are possible for polymerized gel-filled capillaries. The resolving power of the separation depends on the size of the pores, which is based on the composition of the gel. Kits are available to separate fragments into the desired size ranges. Fragment sizes outside the resolution window can possibly be separated, but the separation may not be reliable or reproducible when the gel capability is exceeded.

Fragments can be detected by a variety of mechanisms. Detection utilizing UV absorbance is possible, but the preferred and most common detection procedure is laser-induced fluorescence (LIF). Fluorescence offers improvements over UV detection in terms of selectivity and sensitivity. In addition, the detection limits for fluorescence are two to three orders of magnitude better than those for UV. Although DNA is intrinsically fluorescent, the background fluorescence and complex laser spectroscopy required preclude routine use. The most common way to label DNA is described in the section above on fluorescent protocols for RNA and DNA quantitation. This system is widely employed because of its simplicity (the dyes are added to the sample or into the reaction buffer) and effectiveness. The advantages of CE include speed of analysis, sensitivity using minimum sample volumes, and the potential for automation. These are achieved mainly by the inherent miniaturization of the gel. Automated

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systems allow robust analysis of the quality, quantity, and fragment size of both RNA and DNA. CE applications have been especially important for evaluating the integrity of RNA because of the instability and progressive degradation of RNA caused by ubiquitous RNases, and new technologies that compare the ratios of 28S and 18S are improving the capabilities of these procedures.

FILTER HYBRIDIZATION AND IN VITRO LABELING OF PROBES

Introduction

Hybridization techniques were used early in molecular biology to identify individual nucleic acids and to estimate the degree of similarity between species. Hybridization is widely used in the procedures described in this and other chapters to visualize and identify nucleic acid sequences (see *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩). With the advent of restriction endonuclease digestion of DNA and electrophoretic separation by molecular mass, hybridization using labeled probes provided a way to visualize the organization of genes within a specific genome.

The hybridization techniques described are dot and slot blotting, Northern blotting, Southern blotting, in situ hybridization, and fluorescent in situ hybridization (FISH). All these techniques rely on the use of nucleic acid probes. Probes are oligonucleotides with specific DNA or RNA sequences that have been labeled with radioactive, fluorescent, chemiluminescent, chemical tags or enzymes (reporter molecules). Hybridized probes bind to complementary sequences on the target nucleic acids and are used to visualize and characterize targets, as described below.

Dot and Slot Blotting

Dot blotting is the simplest and quickest of the hybridization techniques. The nucleic acids are directly applied to a support membrane, which may be a nitrocellulose or nylon membrane, without prior separation of the nucleic acid species by agarose gel electrophoresis. The nucleic acids are spotted onto the filter using a micropipettor or an apparatus such as a dot blot or slot blot apparatus. This consists of a membrane frame with a membrane sandwiched in between the two pieces of the frame. The bottom frame plate is connected to a vacuum manifold, and the top piece of the frame has slots through which the nucleic acids are loaded. The samples are loaded under vacuum and pulled through the membrane by vacuum, with the nucleic acid binding to the membrane, and then the filter is air-dried. The nucleic acids are fixed to the filter either by heating to 80° for nitrocellulose membranes or by exposure to UV light for a predeterrmined time for nylon filters. Hybridization with a labeled probe provides confirmation of the identity of the nucleic acid but does not provide any information about the number or sizes of the species. The nucleic acid species of interest can be quantitated by spotting known concentrations of the purified nucleic acid on the filter and comparing the signal generated by the unknown samples with those of the standard preparations.

Southern Blotting

Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane. Small, single-stranded DNA probes can then be used to visualize and identify the DNA species of interest. Southern blot analysis is based on a transfer and immobilization methodology developed in 1975, coupled with the electrophoretic separation of fragmented DNA. More specifically, the procedure typically is used to identify specific nucleic acid sequences in the context of a defined genetic topography, such as a restriction endonuclease map. The position of genes within the viral genome can be accurately mapped using a variety of restriction endonucleases in combination with Southern blot analysis. The procedure requires that DNA be obtained in sufficient quantity for analysis. Fragmented DNA is separated according to size using agarose gel electrophoresis. Double-stranded DNA fragments must be denatured before they are transferred and immobilized on a membrane by capillary action. The immobilized DNA is then cross-linked to the filter, which may be composed of nitrocellulose or nylon, as described above. However, the use of positively charged nylon membranes eliminates the need to fix the DNA to the nylon membrane. Nitrocellulose membranes are more fragile and may be probed up to 3 times with separate probes. Nylon membranes are more robust and may be probed 10 to 12 times, but they may present more background noise, particularly when they are used with chromogenic probes.

Northern Blotting

Northern blot analysis comprises a series of steps for the separation, transfer, and immobilization of RNA in a manner similar to the treatment of DNA using Southern blot analysis. Denaturation of the RNA is required to reduce secondary structure to ensure that the RNA separates in the agarose uniformly according to length. Denaturation of RNA is accomplished either prior to electrophoresis using glyoxal or dimethyl sulfoxide (DMSO) or during electrophoresis by means of gels that contain formaldehyde. Transfer is achieved in a manner identical to that used for Southern blotting. However, in the case of Northern blotting, it is unnecessary to denature the RNA prior to transfer because denaturation is accomplished before electrophoretic separation of the RNA species. The immobilized RNA is cross-linked to the membrane in a manner similar to the cross-linking of DNA.

In Situ Hybridization and Fluorescent In Situ Hybridization (FISH)

Hybridization of a nucleic acid in situ classically refers to determining the location of that nucleic acid sequence in its natural state—in tissue, in individual cells, or on a chromosome. In situ hybridization probes are designed to bind to complementary nucleic acid sequences, whether they be DNA or RNA. The purpose of these hybridization procedures is to discover where in a

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tissue a gene is being expressed, in which case the target is RNA, or to map a specific DNA sequence to its location on a chromosome, in which case the target is DNA.

Chromosome mapping of DNA sequences is accomplished by chemically attaching silver grains to the probe sequences and then counting the density of the grains in a metaphase chromosome spread. Although, historically, these procedures worked well, sensitivity was always an issue. The solution was to use a reporter that was more sensitive and safer than the other reporters, namely, fluorescence used in the technique of fluorescent in situ hybridization (FISH). FISH has an additional benefit in that the different colors available in fluorescence afford the ability to observe multiple hybridization events simultaneously, a feature not available with other detection systems.

Detection of DNA and RNA in Hybridization Assays Using Labeled Probes

Visualization and location of individual nucleic acid species of interest are achieved by the specific hybridization of DNA or RNA probes that are labeled for easy visualization. The filter or sample (fixed cells or tissues in the cases of in situ hybridization and FISH) is incubated with the labeled probe at an appropriate temperature and salt concentration that allows hybridization of desired stringency. This is followed by washing with buffers of varying detergent and salt concentrations and at varying temperatures in order to minimize background signal due to nonspecific hybridization. The labeling and types of probes are discussed below.

Probes can be RNA probes generated in vitro or DNA probes, either double-stranded fragments, plasmids, or single-stranded oligonucleotides containing moieties to facilitate detection of fragments that contain portions of the gene of interest. Probes can be labeled with radioactive tracers such as ^{32}P or ^{35}S by incorporation of a labeled nucleotide in the probe sequence or with a nonradioactive label such as biotin by incorporation of a modified base, such as adenine monophosphate linked to biotin. Radioactive probes are visualized with X-ray film placed over the blot. Biotin-labeled probes are detected with a conjugate of streptavidin–alkaline phosphatase. An enzymatic reaction is run with alkaline phosphatase and a substrate that yields an insoluble colored product at the site of the probe. Variations on nonradioactive probes utilize other modifications to the DNA and linked antibody–alkaline phosphatase, as well as chemiluminescent probes that are detected on film.

Nucleic acids can be synthesized and manipulated by either enzymatic or chemical means. These same systems can be used to modify nucleic acid structure and to introduce foreign moieties to create unique molecules that can provide an advantage to the detection of limiting viral nucleic acids against a background of host nucleic acids. The chemical synthesis of nucleic acids and their purification has become routine, and high-quality synthesis and purification are commonly achieved. Moreover, larger segments can be synthesized, and when even larger segments are required, the subsections can be designed for concatenation and ligation.

Custom synthesis of DNA oligonucleotides is readily achievable in the laboratory using commercially available reagents and equipment. Alternatively, probes can be custom ordered from numerous commercial providers. Size-exclusion procedures for purification generally are used to eliminate incomplete oligonucleotides. RNA oligonucleotides also may be chemically synthesized or generated in vitro using complementary cloned DNA fragments under the control of various prokaryotic RNA polymerase promoter sequences. The use of DNA probes is much more common, but there may be some applications in which the increased association of RNA–RNA or RNA–DNA hybrids is advantageous.

The principal procedures of labeling DNA are direct labeling using a kinase reaction to attach a labeled nucleotide to the end of each DNA strand, by incorporating labeled nucleotides into a nicked DNA by utilizing the DNA repair function of the Klenow fragment of *Eschericia coli* DNA polymerase I enzyme (nick translation), and by PCR. This last procedure generates a relatively higher yield of internally labeled probe because each round of thermal cycling doubles the amount of labeled probe, whereas the former procedures result in a ratio of less than one probe molecule per template molecule. The PCR procedure also is used to generate unique probes with a variety of moieties located at the termini.

NUCLEIC ACID SEQUENCING

Introduction

The first DNA sequencing procedure, described in 1977, utilized chemical cleavage to specifically introduce chain breaks in a DNA sequence (Maxam and Gilbert sequencing). The procedure proved to be of significant utility in the early years of molecular biology, but it has not been used to perform high-volume sequencing and therefore is not discussed in detail here. The majority of sequencing performed today is based on the dideoxysequencing procedure, also described in 1977 (Sanger sequencing). This procedure fundamentally changed sequencing by exploiting the enzymatic specificity of polymerases that introduce strand interruptions at specific bases. This is the most widely recognized sequencing procedure and is considered a routine assay in molecular biology laboratories. Innovations in instrumentation, sample preparation and collection, data management, data analysis, and sequence assembly have relied on this sequencing procedure as their fundamental sequence generator.

High-throughput sequencing takes all the elements of the sequencing procedures and applies them to a mass collection of sequence data, typically for larger genomes, but high-throughput sequencing certainly may be used for smaller projects as well. Obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and data finishing. The technology to achieve these individual objectives includes the instrumentation, disposables, protocols, and procedures.

Sequencing Reaction

The dideoxysequencing procedure takes advantage of specificity of the Klenow enzyme to introduce chain-terminating nucleosides, called dideoxynucleotides, intermittently during the polymerase extension process. The sequencing of each sample requires four separate reactions (one for each base). The resulting mixture of various nucleotide chain lengths is then separated

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on the basis of individual molecular masses. The incorporation of radioactively labeled nucleotides during the sequencing reaction permits the detection of the nucleotide chains.

Improvements in biotechnology have led to the discovery of more robust enzymes with high fidelity, improved stability, and other attributes that have led to longer reads and improved sequence fidelity. These improvements have made possible the introduction of cycle sequencing, which is now commonly used. The principle of the cycle sequencing procedure is a combination of Sanger sequencing and aspects of PCR amplification, whereby dideoxynucleotides are incorporated into the amplified DNA. Cycle sequencing leads to a higher concentration of labeled fragments covering a wider range of sizes than does Sanger sequencing, leading in turn to a higher read length.

Separation Procedures for DNA Sequencing Fragments

The previous sections of this chapter deal with the treatment of intact DNA and RNA molecules; the following sections address the challenges of separating the fragments that result from the sequencing reactions, notably slab gel sequencing and capillary electrophoresis. Subsequent sections address detection technologies and sequence integrity.

Slab Gel Sequencing

Polyacrylamide gel electrophoresis, frequently referred to as slab gel electrophoresis, was the first separation mechanism employed for the separation of DNA sequencing fragments. As described above, the electrophoretic separation of DNA fragments is driven by the size of the fragments in the reaction mixture. However, for slab gel sequencing the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. In addition to the polyacrylamide in the gel, a denaturant such as urea is frequently included to ensure denaturation of the fragments. Until the implementation of multicapillary sequencing systems, the separation power and throughput of slab gel separation mechanisms were often considered state of the art.

Capillary Electrophoresis Sequencing

As noted above, capillary electrophoresis offers significant advantages over gel-based separations. However, as with slab gel sequencing, the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. Multicapillary systems that utilize 8 to 384 capillaries are commercially available. These systems are the primary systems used for large-scale DNA sequencing, and, theoretically, they yield more than 1.1 billion base pairs of DNA sequences per year.

Detection

RADIOACTIVITY

The first detection strategies for DNA sequencing reactions utilized radioactive isotopes such as ^{32}P or ^{35}S, primarily because these were practical for gel separations. The advantages are that detection is universal, low limits of detection are possible, mobility shifts are eliminated, and fidelity differences for the DNA polymerases do not occur. Disadvantages include the high disposal and safety costs, the inability to multiplex (ultimately limiting throughput), and the need for 24 to 36 hours of exposure time (i.e., no real-time detection).

FLUORESCENCE

Fluorescence dyes have largely replaced radioactive isotopes as detection tools during DNA sequencing, mainly because they do not have the disadvantages of radioactive probes. Because the dyes can be discriminated by means of their emission maxima, multiplexing is possible, so four sequencing reactions per sample can be replaced by a single reaction using four different labels. Thus a single lane can be used rather than the four separate lanes that were necessary with radioactive probes. Additional advantages are higher throughput and automated data collection in real time.

MASS SPECTROMETRY

Mass spectrometry (MS) has revolutionized the field of biochemistry and has significant potential in the area of nucleic acid sequencing. Soft-ionization techniques such as electrospray ionization and matrix-assisted laser desorption–ionization have expanded the potential application of MS to DNA sequencing. MS offers some advantages over other detection methodologies, including speed of fragment detection (signal acquisition is in the range of microseconds versus hours for conventional approaches) and accuracy (e.g., the molecular mass of each fragment can be determined with a high degree of accuracy). The Sanger procedure makes use of mass differences of the fragments generated as part of the polymerization reaction. MS is sufficiently precise to resolve fragment sizes that differ by only one base pair. Unfortunately, the sensitivity of MS detection suffers as fragment length increases, and the 100-base-pair barrier has yet to be crossed.

More recently, other sequencing technologies have emerged that are based on massively parallel sequencing techniques that attempt to achieve low-cost sequencing. These techniques are based, for example, on solid-phase sequencing or they make use of highly parallel and miniaturized pyrosequencing, which is described in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩.

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Sequence Integrity

A prerequisite for automated data collection and interpretation is that the data must be of good quality, which means minimizing human intervention and allowing the system to make base identifications following detection steps. It is a critical step to ensure accurate base identification by minimally sequencing both strands of the DNA several times. In addition, other tactics may be employed, such as using primers at different sequence positions, which can improve the accuracy of the developed consensus sequence. This task can be facilitated by the use of specialized software packages that are commercially available. More recent technology developments have produced alternative sequencing platforms that are more amenable to large-scale sequencing projects. These techniques include array-based platforms on which short stretches of target are sequenced on a chip that supplies raw data to sophisticated computational programs that reconstruct the sequence. Other sequencing approaches have been developed for the rapid sequencing of short nucleic acid sequences such as oligonucleotides of short PCR products. These technologies include MS-based and pyrosequencing platforms, the latter of which is described in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩.

⟨1127⟩ NUCLEIC ACID-BASED TECHNIQUES—AMPLIFICATION

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid–Based Techniques—General* ⟨1125⟩. The current chapter covers major techniques that result in amplification of targeted nucleic acid sequences. The most common NAT assay is the polymerase chain reaction (PCR), which was first described by Kary Mullis. This procedure has been further refined to amplify a DNA fragment starting from RNA (reverse transcription-PCR, or RT-PCR). Initially, PCR was used in a qualitative manner to amplify and detect DNA molecules because its exquisite sensitivity paired with its high specificity made it a useful tool for the detection of nucleic acid targets. Since its inception, the number of PCR applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. In addition to the changes and improvements to the original design of the PCR procedure, alternatives to PCR are techniques used to amplify target nucleic acids to generate RNA instead of DNA amplicons. The most commonly used techniques are nucleic acid sequence–based amplification (NASBA) and the transcription-mediated amplification (TMA) which are described here in detail. In contrast to PCR, which relies on incubating the sample at three different temperatures, NASBA and TMA are based on isothermal conditions.

In addition to amplification of the target nucleic acid, the amplification step also can be directed at the signal used for detection (signal amplification). The most commonly employed technique is the branched DNA (bDNA) assay, in which the signal, typically a fluorescent probe that binds to the target sequence, is amplified. The bDNA assay is used predominantly for viral nucleic acid detection and quantitation.

This chapter describes the main assay components necessary for a PCR procedure and includes a discussion of the general optimization of PCR assays. The various PCR assay formats, including PCR, nested PCR, and RT-PCR are covered, and a discussion of the detection of the resulting amplicons follows. Although all these assays are essentially qualitative procedures, they can be modified for semiquantitation, and the various modifications are described. For accurate and reliable quantitation, real-time PCR has now replaced the methods listed above; real-time PCR and real-time RT-PCR are described in the *NAT Assays* section. The same section includes a discussion about probes and dyes that are an essential component of real-time PCR and the methods of quantitation using the generation of standard curves. The next PCR technique discussed is multiplex PCR, which is used for simultaneous detection of multiple targets or for normalization of assay results. Apart from PCR, the major alternative NAT tests that are used routinely, primarily in blood screening and clinical diagnostic screening are NASBA and TMA. The final technique described is whole-genome amplification, wherein the complexities of amplification require modifications to the PCR procedures. The chapter concludes with a discussion about the evolution of instrumentation used in NAT assays and the quality assurance and quality control issues associated with NAT because this is probably one of the most highly regulated biological techniques, especially when applied to blood screening.

ASSAY COMPONENTS

Enzymes

The essential components for NAT assays—polymerases, reaction buffers which include desoxynucleotides, ions, primers, probes, and fluorescent dyes—can be chosen from a broad selection of commercially available NAT reagent kits and vendors. Polymerases suitable for NAT applications can, in principle, be grouped into *Taq* DNA polymerases or DNA I polymerases from other *Thermus* species that are polymerases with features that are similar to those of *Taq* DNA polymerase. In addition, so-called proofreading polymerases are available (e.g., from *Pyrococcus* species) that display a 3′–5′ exonuclease activity capable of removing wrongly incorporated DNA bases from the growing DNA strand under amplification conditions. *Taq* DNA polymerase is the standard NAT enzyme and is the one most often used in NAT assays. Modifications of *Taq* DNA polymerase, such as deletions of the 5′–3′ exonuclease domain (Klenow fragment, Stoffel fragment) or point mutations for improved incorporation of dideoxynucleotides are also employed (e.g., for PCR-based sequencing reactions). Proofreading DNA polymerases or mixtures of *Taq* DNA polymerase with a proofreading polymerase are used if either fidelity of the NAT product is critical (e.g., for DNA cloning experiments) or longer NAT products are to be amplified. For RT–PCR, a reverse transcriptase is necessary to first convert the RNA target to copy DNA (cDNA) that can subsequently be amplified. For TMA reverse transcriptase with an RNAse H activity is needed to convert the RNA target to double-stranded template DNA, while for NASBA exogenous RHAse H

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is added to the reaction mixture. Depending on the reaction environment, two types of enzymes can be used to generate cDNA: a reverse transcriptase isolated from retroviral sources or a DNA polymerase that can function both as reverse transcriptase and DNA polymerase. Finally, chemical modification of the polymerase, resulting in an inactive enzyme at temperatures below 90°, is now typically used to prevent mispriming of templates at sub-optimal temperatures (see section on *Assay Optimization*).

Reaction Buffers

Reaction buffers vary with respect to ion composition, pH, and additives and are sometimes specifically adopted for particular applications such as multiplex PCR, real-time PCR, RT–PCR, TMA and NASBA. An important component of the reaction mixture is Mg^{2+} ions, or, in the case of polymerases with both reverse transcriptase and DNA polymerase functions, such as *Thermus thermophilus* (*Tth*), Mn^{2+} ions. Other additives that enhance the sensitivity and specificity of the assay may be present. The concentration of the four deoxynucleotide triphosphates (dNTPs) must be optimized.

Primers

Primer sets are oligonucleotides with sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Synthetic oligonucleotide primers for both standard PCR and for real-time or quantitative PCR are designed for the specific recognition of and binding to a single DNA or RNA sequence. Such specificity is achieved through design that involves both the length and the sequence of the primers. Length and sequence specifications have separate criteria that must be simultaneously met in order for the primers to perform properly. The length of a primer is a statistical issue that relates to the issue of the minimum length of a specific sequence necessary to guarantee that the desired target sequence is unique, regardless of the size or complexity of the genome. As an example, in the case of the human genome, with its 3.2 billion DNA bases, that length is 17 bases. For this reason the vast majority of PCR primers are between 20 and 25 bases long. The specificity of a primer should be determined by comparison with sequences in all known databases. Tools available on the Web facilitate such comparisons.

In terms of primer sequence, the issues to consider are T_m (the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded) and secondary structure. Every DNA has its own characteristic T_m, determined by length, sequence composition, and reaction environment. PCR primers are designed to bind to a perfectly complementary DNA sequence via guanine-cytosine (G-C) and adenine-thymine (A-T) base pairing. The T_m of the two PCR primers used in a reaction should be as close as possible. In terms of secondary structure, the formation of secondary structures by intra- or intercomplementarity should be minimized. Interaction between different primers can result in primer–dimers that will compromise assay sensitivity and specificity. All of the design issues presented are accounted for in any one of the dozens of primer design software packages that are available and can be found on the Internet.

Assay Optimization

NAT assay optimization is necessary for successful amplification that is sensitive and specific. Parameters that should be optimized include the thermocycling conditions, both temperatures and cycling times (that depend to a large extent on the target, primer, and probe sequences), concentrations of template, concentrations of NAT reagents, sample matrix and the number of amplification cycles. In the case of multiplex PCR, a compromise among elements of the reaction conditions is usually necessary because of the difficulties in optimizing the conditions for all the primer and probe sets. Recent changes have been made to improve sensitivity and specificity of NAT assays. One change is hot-start PCR, in which the addition of one of the essential components of the NAT assay, typically the DNA polymerase, is temporarily withheld. When this occurs during reaction setup, the initial nucleic acid denaturation step prevents nonspecific amplification due to mispriming at suboptimal temperatures. Early hot-start procedures made use of wax barriers that effectively separated essential components into two liquid phases that were mixed only when the wax melted. However, this procedure has been replaced by two important hot-start technologies that do not require physical separation of the components by inconvenient additional handling steps. In the first procedure, antibodies directed against the DNA polymerase are complexed with the enzyme and lose their binding avidity at elevated temperature at the start of the reaction. The second procedure uses chemical modification of the polymerase, resulting in an inactive enzyme. At temperatures above 90°, typically in the first denaturation step, the modifier dissociates from the enzyme, and the enzymatic activity is restored. The advantage of an antibody-mediated hot start is the immediate release of enzyme activity at the start of the reaction by a very short heat incubation step. However, antibody-mediated hot-start chemistries tend to be less stringent when compared with chemically activated enzymes if there is a large excess of active polymerase molecules.

NAT ASSAYS

This section describes the basic techniques of PCR, nested PCR, and RT-PCR and procedural modifications that allow semiquantitation.

Polymerase Chain Reaction

The PCR technique is based on a three-step process: denaturing double-stranded DNA (dsDNA) into single strands (ssDNA), annealing primers to the ssDNA, and enzymatic extension of primers that are complementary to the ssDNA templates. Each step is usually carried out at a different temperature. By cycling the temperature steps many times (usually 30 to 45 times), a billion-fold amplification of the target nucleic acid can be achieved, but the optimal number of cycles should be determined empirically. In some cases, especially where sensitivity is more important than false positive results due to excessive cycling,

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such as in blood screening, extra sensitivity can be gained by increasing the number of cycles to 60 to ensure that extremely low levels of target are detected. In a typical reaction, PCR product (amplicon) doubles at each cycle of amplification (exponential amplification). The increase in amplification in the early cycles follows a sigmoidal curve. In later cycles, the concentrations of the template strands and amplicons favor template strands re-annealing instead of PCR primer annealing to the template. At this point the concentration of the PCR product no longer doubles after each cycle, and the curve begins to plateau. A thermostable enzyme such as *Taq*-polymerase is a prerequisite because temperature cycling at 95° (the typical temperature step used to denature double-stranded templates) would inactivate a thermolabile polymerase.

NESTED PCR

An early variation of the PCR assay was nested PCR, which was designed to increase the assay's sensitivity and specificity. In this procedure amplicons from the initial PCR reaction are subjected to a second round of amplification using a different set of primers. This set of primers is specific to the amplicon sequence but is within the first set of primers (nested primers). The advantage of amplification with two sets of target-specific primers is increased specificity (any nonspecific amplification during the first amplification round would be reduced) and increased sensitivity (due to initial amplification of the target in the first amplification round). In addition, amplification of a product of the expected size is taken as confirmation of the presence of the target. However, a major drawback of this procedure is the high likelihood of cross-contamination due to the increased manipulation of amplicons generated in the first round of amplification. The use of highly specific primers and probes and the optimization of reaction conditions have resulted in the diminished applications of this procedure for routine testing, but the procedure is sometimes used for samples that are difficult to amplify by conventional PCR.

RT-PCR

In amplifying RNA targets, analysts prepare cDNA before the amplification step (RT-PCR). One-step and two-step RT-PCR procedures are available. In one-step RT-PCR the reverse transcription of RNA into cDNA and the subsequent amplification step are carried out in a single reaction without intermediate procedures. Therefore the reaction mixture for one-step RT-PCR includes the gene-specific amplification primers that are used for both reverse transcription and amplification. The advantage of this procedure is the overall reduction in handling time, increased throughput, and reduced contamination risk because reopening the reaction vessel is not necessary. In contrast, in two-step RT-PCR the reverse transcription and amplification are performed as two separate steps. In general, random primers or oligo-d(T) primer rather than gene-specific primers are used for the reverse transcription step. An aliquot of the cDNA synthesis reaction is then transferred into the NAT reaction for subsequent amplification. The advantage of this procedure is the standardization of the reverse transcription reaction, which can be used as a single source for the analysis of multiple transcripts in gene expression analysis.

DETECTION OF AMPLICONS

Following amplification, analysts can employ a variety of procedures for detection of the amplicon as described in detail in the general information chapter, *Nucleic Acid–Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. These include agarose gel electrophoresis with ethidium bromide or other dyes, capillary electrophoresis, and laser-induced fluorescence and hybridization followed by chromogenic detection such as streptavidin horseradish peroxidase detection, chemiluminescence, or fluorescent detection using labeled probes.

Quantitation—The original PCR and RT-PCR assays were qualitative and detected amplicons at the end of the reaction. Such detection is not easy to quantitate because at this stage the amplification is in a plateau phase at the end of the assay, and the amount of amplicon is not necessarily directly related to the quantity of the starting template. Several approaches have been deployed to attempt to overcome the shortcoming of PCR to produce reliable, quantitative results. Initial attempts at quantitation relied on assessing the amount of amplified DNA during the early or exponential part of the assay, but this procedure was fraught with problems because the aliquots had to be taken from the reaction mixtures at regular intervals, thus greatly increasing the risk of cross-contamination. One of the earliest and most straightforward approaches to quantifying PCR products was to measure the amount of amplicons that were generated during the exponential phase of the reaction by comparing this to a serially diluted external control. Several aspects, including variability in sample preparation and variations in reaction conditions, however, hampered this approach. Because of the exponential amplification of NAT procedures, even small errors or variances can lead to distinct differences.

Compared with dilution procedures, competitive PCR proved to be a much more precise approach to achieving reliable estimates of the originally present target molecules. This procedure relies on the simultaneous co-amplification of a specific target sequence in the presence of increasing concentrations of an exogenous target molecule (control) which shares the primer binding sites with the target sequence but whose sequence is slightly modified or shortened in order to facilitate discrimination from wild-type amplicons. In addition, the concentration of the control is known. The close sequence homology and similar size of the control and target amplicons are designed to ensure that the template and internal control are amplified with comparable efficiency. The relative strength of the amplicon bands of template and control can be assayed, for example, on ethidium bromide–stained agarose gels, giving a relatively precise quantitation of the wild-type target. A drawback of this approach is that the internal control and the template should be present in the reaction in approximately the same quantity in order to yield correct results. The development of real-time, quantitative PCR has eliminated the variability associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products.

REAL-TIME PCR AND REAL-TIME RT-PCR

Although gene quantitation by quantitative PCR was a widely used procedure, its applications were expanded by the advent of real-time PCR and real-time RT-PCR. Real-time PCR displays the same advantages as standard quantitative PCR—sensitivity, specificity, and a wide dynamic range—but the real-time procedure offers the additional advantage of requiring no post amplification processing because it combines amplification and detection in a single step. Real-time PCR collects data

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throughout the amplification process by measuring a fluorescence signal created as amplification progresses. A multitude of fluorescence chemistries allows the correlation of generated PCR product to fluorescence intensity. In principle, fluorescence intensity will increase with every cycle performed. Once the intensity is greater than background fluorescence, the so-called cycle threshold (C_t) value is achieved. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. The C_t value is inversely proportional to the number of target molecules in the sample and thus provides a means to quantitate the amount of target in the starting material (i.e., the greater the number of target molecules present, the lower the C_t value).

The reaction conditions for real-time PCR applications have to take into account the presence of the probe(s) and will require optimization. The most commonly used probes currently are hydrolysis probes, although hybridization probes are an alternative. In most cases, the amplification and detection steps can be combined into a two-step cycling reaction, but these conditions have to be optimized. In contrast, DNA-binding dyes which may also be used for amplicon detection require separation of the annealing and extension steps since the dye binding occurs during the extension step which is usually done at 72°.

A fluorescent DNA intercalating dye is used for detection of the PCR product in real-time mode. This dye emits light when bound to double-stranded DNA and the subsequent increase in fluorescence can be detected by real-time PCR instruments. Dyes that bind to dsDNA bind not only to the specific PCR product but also to artifacts such as nonspecific PCR products and primer–dimers. Analysts have observed substantial differences in the specificity of dsDNA-binding dyes in use with real-time PCR kits. Therefore, some analysts recommend verifying the presence of a single PCR product by gel electrophoresis to determine the correct size of the PCR product. Also, a melting curve analysis is advisable to ensure the absence of artifacts that could contribute to the fluorescent signal and thereby lead to misinterpretation of quantitative data. Alternatively, sequence-specific labeled probes can be employed. A wide variety of fluorescence-labeled probes and primers exist for use in real-time PCR and are described in the next section.

Real-Time PCR Probes—The difference between conventional PCR and real-time PCR is the presence of a third chemically synthesized oligonucleotide, the probe, which, for the most basic hybridization probes, contains some type of reporter molecule, usually a fluorescent molecule or fluorophore. Non-nucleic acid materials can be added to chemically synthesized DNAs that are then incorporated into oligonucleotide probes for real-time PCR. Other applications include hybridization probes such as those used for fluorescence in situ hybridization (FISH) and microarrays and probes designed to capture other nucleic acids. A challenge arises in using fluorescent probes for real-time PCR because the unbound or free probe is not removed before detection, thus requiring a means to distinguish between signal obtained from bound and free probe. In contrast, FISH assays involve washing away free probe following hybridization.

All of the issues associated with primer design for conventional PCR apply to real-time PCR primers as well as to the probe sequence. As a general rule only two additional considerations apply to the probe sequence. One of these is thermodynamic, and the other specifically concerns the reporter moieties themselves. Thermodynamically, a good probe molecule that is designed to bind in the sequence somewhere between the two PCR primers will have a T_m that is about 5° higher than that of the two primers. In the large majority of cases the amplicon will be between 100 and 500 DNA bases in length, although for real-time PCR a smaller amplicon between 100 and 150 bases long results in a more efficient reaction. Thus it is rarely a problem to find a sequence inside a PCR amplicon that meets the necessary criteria.

Current probe designs overcome the problems of background from unbound probe using simple hybridization probes. In the original design, two probes that hybridize to adjacent sequences on the target nucleic acid are labeled. The reporter moiety is a fluorescent molecule attached to the 3′ end of the upstream probe sequence, and a second fluorescent molecule is attached to the 5′ end of the second probe. Excitation of the 5′ fluorophore with light energy of the proper wavelength results in absorption of that energy, followed by emission of light energy of a slightly longer or less energetic wavelength (Stoke's Law). This emitted energy then excites the 3′ fluorophore if it is close enough to the emitter and compatible with it in the sense that the emitted energy from the 5′ fluorophore can excite the 3′ fluorophore. When this occurs, the observed fluorescent light wavelength will be that of the acceptor molecule and not that of the donor. Fluorescence absorption and emission spectra are readily available for all of the commonly used fluorophores, and the only applicable rules are that the two fluorescent molecules must be fewer than 40 DNA bases apart and that the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Thus hybridization of the two probes, also known as hybridization probes or FRET probes (Fluorescence Resonance Energy Transfer), results in the emission of a fluorescent signal by the acceptor, and the latter signal can be detected. In the absence of hybridization, the probes are sufficiently separated in solution so that energy transfer cannot occur, and only background fluorescence is emitted by the donor.

Issues of fluorophore compatibility have been resolved by the increased use of a special class of molecule called a quencher. Quenchers are fluorescent molecules that absorb fluorescence energy over a wide range of wavelengths. Instead of re-emitting that energy as light they simply dissipate it as heat. Thus, if a quencher molecule is placed at the 3′ end of a probe and a fluorophore at the 5′ end, the probe will remain dark even when excitation energy is present so long as the molecule remains intact (hydrolysis probes). These probes utilize the 5′ nuclease activity of the DNA polymerase to hydrolyze a probe bound to its target amplicon. Cleavage results in separation of the reporter and quencher and permits fluorescence of the reporter. This reduces much of the work of optimization of the assay conditions (since only a single probe is used) and background noise generated with two probes.

A variation on hydrolysis probes involves placing the reporter and quencher molecules on a single oligonucleotide that is constructed so that, in the unbound state, the quencher and reporter are in close proximity, resulting in efficient quenching of the reporter. When the probe hybridizes to its complementary sequence on the amplicon, the probe undergoes a conformational change that forces the quencher and reporter apart, permitting fluorescence of the reporter. A variation on these kinds of probes is a combined primer and probe in which, again, the quencher and reporter are in close proximity in the native probe, thus resulting in no signal. Priming and subsequent elongation of the primer–probe results in hybridization to the newly synthesized DNA strand, causing spatial separation of the quencher and reporter and resulting in the generation of a signal.

Probe Labeling—Modern synthetic oligonucleotide modification chemistries permit the manufacture of oligonucleotides with non-nucleic acid materials. Placement of modifications is carried out in one of two ways: during synthesis or after synthesis. For the former, modifications are constructed in such a way that they behave like the four DNA or RNA bases that are routinely

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placed in the sequence. The modification is then presented in the desired location during the synthesis as if it were just another base in the series. In the latter, usually employed when more than one modification occurs, the synthesis contains a linker, such as an amino group, to which the desired modification is then attached. This process is often called "hand-tagging."

Perhaps the best-known example of hand-tagging is the conventional dual-labeled probe used in real-time PCR. The quencher is placed at the 3′ end of the sequence during synthesis, and the fluorescent reporter molecule is hand-tagged to an amino modification at the 5′ end of the sequence after the synthesis is finished and has undergone purification. Some modifications, such as biotins, are designed so that multiple modifications can be carried out in a single synthesis. Thus, it is possible to modify a synthetic DNA or RNA sequence to contain a number of different non-nucleic acid molecules. A cost is associated with such modifications insofar as alterations often are achieved with a loss of mass due either to an inherently lower efficiency of modifications to bind to the oligonucleotide as compared with standard DNA or RNA bases or to the requirement that the synthesis must be purified before modification, after modification, or both.

The benefits of modifying synthetic DNAs or RNAs usually outweigh the costs. The standard, quenched, dual-labeled, real-time PCR probe has permitted precise quantification of gene expression. Fluorescently labeled DNA oligonucleotides are also essential components of in situ hybridizations and microarrays. Some modifications confer increased thermal stability when synthetic DNAs or RNAs are hybridized to complementary DNAs or RNAs by comparison with unmodified DNA–DNA and DNA–RNA duplexes. These analogues include peptide nucleic acids, 2′-fluoro N3-P5′-phosphoramidates, and 1′, 5′-anhydrohexitol nucleic acids. Although such analogues succeed to varying degrees in achieving increased thermal stabilities, they fail to provide enhanced target recognition. Another approach is to use base analogues such as locked nucleic acid, which is an analogue that contains a 2′-O, 4′-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and stability.

The modification of a probe typically is governed by its intended use. Generally, fluorescent reporters are used in real-time PCR and for in situ hybridization. The range of available fluorescent reporters covers the spectrum from 517 nm to 778 nm. For hybridization probes, base modifications are preferred because these primarily alter thermodynamic interactions between bases, leading to improved specificity. Amino attachment groups, both with and without C-spacers, are used to attach other modifications to DNA sequences and to attach DNA sequences to solid surfaces such as glass slides. An example is the attachment of biotin molecules to DNA sequences. Biotin forms a strong bond with streptavidin-coated materials such as magnetic beads, allowing capture of specific nucleic acids that may themselves be hybridized to other molecules.

Quantitation—PCR products may be quantified using a standard curve drawn from replicate serial dilutions of a reference reagent or standard for the nucleic acid sequence of interest. The concentration of the nucleic acid in the reference reagent is known. Real-time PCR quantitation based on a standard curve may utilize plasmid DNA or other forms of DNA. However, the efficiency of PCR must be the same for the standards and the target samples. Performing PCR from purified targets can in some cases be more efficient than performing PCR with complex nucleic acid mixtures. The cycle threshold (Ct) values and concentrations of the dilutions of the reference reagent can be used to construct a standard curve from which the concentration of the unknown sample can be estimated. When the assay run conditions have been well standardized and the standard curve for a particular target has been well calibrated, in subsequent assay runs it may be sufficient to co-amplify only two dilutions of a reference reagent (usually dilutions containing known amounts of nucleic acid at high and low concentrations). These dilutions, or calibrators, can then be used to quantitate any unknown samples by comparison of the C_t values.

Multiplex PCR—Multiplex PCR describes the simultaneous amplification of several nucleic acid targets in a single assay reaction. This is a particularly demanding variation of PCR because it requires the use of a single set of reaction conditions for the amplification of multiple targets with different sequence characteristics. Additional complications can arise due to the increased chance of nonspecific amplification products arising from multiple primer interactions. In addition, the differing individual target amplification efficiencies can result in weaker reactions being out-competed by stronger, more efficient reactions.

Both qualitative and quantitative applications of multiplex PCR have been described in the literature, as have multiplex RT-PCR assays. Quantitative multiplex PCR relies on either the generation of multiple standard curves to enable quantitation of each target in the assay, or the inclusion of internal competitor sequences that can be used as calibrants.

Hybridization kinetics of primers and probes may be significantly different, even when designed using the same algorithm. This leaves the analyst with very limited room to optimize reaction conditions. However, optimization may include adjustment of DNA polymerase amount, Mg^{2+} to increase hybridization efficiency, or primer concentration. Especially in real-time PCR, optimization of primer concentration is critical for quantitative co-amplification of target genes. These are contained in the sample at significantly different amounts. Increasing hybridization efficiency of the primer–probe system can be achieved by providing sufficient reagents, such as Mg^{2+}, as well as adding a "molecular crowding" reagent that increases the effective concentration of all reaction components in the mixture. Multiplex PCR is not only used for genotyping applications, but also for quantitative real-time PCR because it offers several advantages over standard single real-time PCR reactions. Some of these advantages are a minimized amount of sample used, increased precision through the use of an internal control (e.g., housekeeping gene) co-amplified with the target gene in the same reaction, no separate pipetting steps, and cost-effectiveness.

Most PCR assays, however, suffer from a common problem—that of minimizing differences in extractions or amplifications between different samples. Multiplex PCR is useful in cases where it is critical to ensure that variability in quantitation of different samples is not due to differences in nucleic extraction or amplification measurements (usually when one measures the production of an mRNA species). Certain precautions and techniques can be employed to minimize these challenges; they are discussed in the next section on normalization of assay results.

Normalization of Assay Results—To minimize the effects of assay variables, analysts sometimes use a relative quantitation procedure that normalizes the target transcript level to a control that can be employed and compared for all samples included in the gene expression study. Probably the most reliable and most frequently used relative quantitation procedure relies on the measurement of "housekeeping" or control genes to normalize the expression of the target gene in a multiplex PCR format. This procedure is preferred because the quantitation of both the housekeeping gene and the target gene are influenced by varying cDNA synthesis efficiencies or the presence of enzyme inhibitors contained in the sample. However, it should be noted that the efficiency of conversion of target RNA to cDNA is not necessarily consistent even within a single-tube reaction but is a function of primer design, target sequence, etc. which may differ between target and housekeeping genes. The selection of appropriate control genes can cause problems because they may not necessarily be equally expressed across all unknown

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samples and may vary under experimental conditions. Normalizing measurements to a set of housekeeping genes in order to avoid the problem of variability may circumvent this concern. Alternatively, analysts can establish a thorough evaluation of housekeeping genes that do not alter gene expression levels under the experimental conditions.

All the NAT techniques described thus far are variations on the PCR assay, which is the most widely used of the NAT techniques. However, isothermal assays that are based primarily on the amplification of RNA are used for routine purposes. This is known as the transcription-mediated amplification (TMA) assay, which is closely related to the nucleic acid sequence–based amplification (NASBA) assay. Both assays are described in more detail in the following section.

NUCLEIC ACID CEQUENCE-BASED AMPLIFICATION AND TRANSCRIPTION-MEDIATED AMPLIFICATION

Both NASBA and TMA rely on in vitro isothermal amplification for detection and amplification of nucleic acids, also referred to as self-sustained sequence replication or 3SR. The major difference between the assays is that NASBA uses three enzymes—reverse transcriptase (RT), RNA polymerase, and RNase H—whereas TMA uses only two enzymes: RT and RNA polymerase. The complete procedure generally is performed at 41° to 42° using two primers. Both NASBA and TMA are especially suited to amplifying RNA analytes, including rRNA, mRNA, pathogens that have RNA as their genetic material, as well as DNA targets.

One of the primers that has a promoter sequence for the RNA polymerase at the 5′ end binds to the RNA target and is extended via the DNA polymerase activity of the RT. The product of this reaction is an RNA–DNA hybrid. RNase H activity then specifically digests the RNA strand of the hybrid, leaving only the cDNA to which the second primer can bind. A complementary strand of DNA is then synthesized by the RT, resulting in a dsDNA molecule with a T7 promoter at the 5′ end. The T7 RNA polymerase then transcribes multiple copies of the RNA amplicon. The RNA copies may undergo the same cycle to create new duplex DNA molecules with a T7 promoter from which many molecules of RNA are transcribed. Thus, unlike the action of PCR, the amplicon amplified in this case is of an RNA species.

Some of the characteristics of this technology are that only relatively short target sequences can be amplified efficiently (around 100–250 nucleotides); it uses a single temperature, which eliminates the need for special thermocycling equipment; the fidelity of the technique is comparable to that of other amplification processes; and the RNA amplicons are exponentially amplified. Carryover contamination is minimized because of the labile nature of the RNA amplicon in the laboratory environment. Containment procedures built into the assay procedure further help to minimize contamination. Detection of amplicons is typically achieved by the use of labeled probes and, in TMA technology, a common method is detection of chemiluminescent signals from hybridized probes that remain intact during the subsequent alkaline hydrolysis step used to destroy free probe.

The NAT techniques described, both PCR and TMA, are optimized for amplifying specific, small fragments of a genome. In cases when whole genome amplification is desirable, such as for mutation analysis or identity testing, modifications of the PCR procedure are necessary in order to ensure adequate sequence representation of genetic loci, as described in the following section.

WHOLE GENOME AMPLIFICATION

Historically, whole genome amplification (WGA) has been performed using modified PCR procedures. These procedures have relied on the nonspecific amplification of the genome using primers that bind under low-stringency conditions to the DNA template. PCR-based approaches differ mainly in terms of the type of primer employed in the reaction: in primer-extension-preamplification (PEP), short 15 base random primers are used in an initial cycling reaction at low stringency to make multiple random copies of segments of the genome. This product is then used as target for the specific PCR reaction. Amplification bias of favorable sequence contexts leading to uneven representation of the genome is the major drawback of this technique. The generation of increasingly shorter fragments during each round of amplification is a further drawback. Another procedure called degenerate oligonucleotide primed-PCR (DOP-PCR) uses tagged primers and low stringency amplification for the first few cycles of amplification followed by an increase in annealing stringency in later cycles. The tagged primers are characterized by defined sequence tags at the 3′ and 5′ ends and a random sequence in the centre of the primer. Under the later, more stringent conditions, the target DNA fragments generated during the first cycles containing the amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA typically employs *Taq*-like polymerases that possess the disadvantage of introducing variations into the amplified DNA due to their relatively low processivity and fidelity which become compounded by the very high number of amplification cycles used in these methods. This may cause problems in downstream applications such as genotyping analysis. These limitations as well as the relatively poor sequence representation of genomic loci inherent to PCR-based WGA can be overcome by an isothermal reaction called multiple displacement amplification (MDA).

The enzyme that is used for MDA comprises a high processivity polymerase with proofreading and strand-displacement activity. The isothermal reaction is performed at 30° without any change in reaction temperature. The reaction starts with the annealing of multiple random primers to the target DNA and elongation of the primers using a DNA polymerase from the *Bacillus subtilis* phage Phi29. Because the polymerase is able to displace DNA strands in a 5′–3′ direction, the polymerase reaction is not stopped when the elongating strands meet downstream DNA strands. The displaced DNA strand serves again as a target for multiple primed elongation reactions so that the DNA template is amplified exponentially in a branched-like manner, yielding high molecular weight DNA with a good representation of the genomic loci. Compared with PCR-based WGA, the error rate is very low. In particular, the mutation rate of repetitive sequence structures is low because of the limited strand-displacement activity of Phi29-polymerase. This permits reliable genotyping of genomic DNA (e.g., SNP analysis, mutation analysis, identity testing, or analysis of case work samples) on different platforms such as real-time PCR or array analysis.

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INSTRUMENTATION

The development of the numerous and varied NAT techniques described in this chapter has been facilitated by the evolution of instrumentation that has served to automate these complex procedures. A general description of the major changes in instrumentation is discussed in this section.

The continuous control of the temperature steps necessary to achieve exponential amplification for PCR assays is carried out by fully automated thermocyclers that consist of a heating block in which the temperature can be rapidly cycled. Temperature changes are induced by water, or more recently, by using the Peltier effect. These instruments may be coupled to a fluorometer apparatus if they are used for real-time PCR analysis. In the latter case certain instruments are equipped with a rotor device that is heated and cooled by air instead of a metal block that typically is used as a heating module. In the case of endpoint PCR, PCR products are usually analyzed according to size on agarose or polyacrylamide gels, or by capillary electrophoresis using fluorophore-labeled primers. They may also be analyzed by an array-based approach or other hybridization procedures.

Because no post-PCR processing or label-separation steps are required, real-time PCR assays are simple to perform, making them useful for high-throughput applications. Real-time PCR instruments combine the properties of a thermocycler and a fluorometer to allow determination of PCR products by fluorescence measurement. In each PCR cycle, either one or several fluorescence readouts are taken to monitor the PCR reaction for generation of amplicons, usually at the extension step of the PCR reaction.

Real-time PCR instruments vary with regard to simultaneous sample throughput (32–384 reaction vessels), sample volume (5–100 µL), excitation source, and detector used. These compositions define the suitable range of fluorescent dyes for multiplex real-time PCR as well as size and heating/cooling principle (see above). The excitation source of real-time thermocyclers is either a laser-based system, halogen bulbs, or light-emitting diodes (LED). Optical filters are used to select the wavelength of interest. In most instruments, the emitted light is detected by a charge-coupled device (CCD) that consists of an array of light-sensitive cells. Light projected onto the CCD is converted to an electric charge, resulting in a signal that is proportional to the light intensity.

The versatility of the PCR assay has resulted in the widespread and diverse use of this technique. With the advent of real-time PCR, it has been possible to design high-throughput instrumentation for automated testing. Similarly, the TMA assay has also been automated. Such technology is used by laboratories doing high-throughput, highly regulated testing, typically blood screening for hepatitis C virus (HCV) or human immunodeficiency virus-1 (HIV-1) because automated tests are ideal in a regulated environment where minimum human intervention is required. The use of NAT in a highly regulated environment has resulted in the development of guidances for managing the quality assurance (QA) and quality control (QC) aspects of testing, as well as the validation of systems and assays as described in the following section.

QUALITY ASSURANCE AND QUALITY CONTROL FOR NAT

This section serves as a general guidance for the development of laboratory- and procedure-specific QC and QA procedures for NAT. Aspects such as waste management, management of radioactive material, or working with hazardous material are not covered. NAT is a technology that offers extreme sensitivity with its ability to generate millions of amplicons from as little as a single nucleic acid template, resulting in a detectable signal. The advantages of this technology can be offset by the necessity of establishing complex assay protocols and the requirement to follow carefully very stringent QC/QA protocols. Deviation from these protocols can cause major problems, such as false positive results due to the contamination of templates by amplicons generated in previous assay runs. Similarly, failure to control inhibitors could lead to suboptimal amplification and possible false negative results. Given the myriad factors that can greatly influence the outcome of a NAT assay, all aspects concerning NAT need to be covered by appropriate and stringent QC/QA procedures. This requires careful facility design, workflow, and selection of equipment suitable to the purpose. Data recording, record keeping, and data interpretation are other aspects that should be covered by QC/QA. Thus, QA for NAT assays includes assay validation, establishment of acceptance criteria and specifications, and adherence to good manufacturing/laboratory practices. These aspects are also described in this section. In addition, reference should be made to other published guidelines such as the ICH Guideline *Validation of Analytical Methods: Methodology* (Q2B) and the NCCLS Guidelines.

Laboratory QC/QA

An NAT laboratory should be designed and operated in a manner that prevents contamination of reactions with products from previous amplifications (carry-over) as well as cross-contamination between samples. Historically, the application of PCR required strict separation of the various steps of the assay in order to prevent cross-contamination of PCR by amplicons. This was necessary because early procedures for analysis of PCR products involved the transfer of the product, which potentially could lead to contamination. Therefore, in an open system the best measure to prevent contamination has been the strict separation of working areas for individual process steps. This includes individual areas for template preparation, master mix setup, distribution of the master mix to individual reaction wells and addition of template, space for cycling the PCR assays and, optionally, a separate work space for PCR product analysis. These requirements are not necessary with closed systems. With both open and closed systems it is still necessary to take additional precautions. These safety measures include UV illumination of work spaces overnight to inactivate residual DNA by crosslinking. In case of contamination, laboratory benches and pipettes can be decontaminated by cleaning with a 10% solution of commercial bleach, which usually contains about 5% sodium hypochlorite, taking appropriate safety measures such as wearing gloves and eye protection. Afterwards, benches and pipettes should be rinsed with distilled water. A unidirectional workflow will reduce the opportunity for contamination to occur. Also, no materials, supplies, or equipment should be exchanged between designated working areas or rooms.

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Equipment QC/QA

Other good laboratory practices that are related to the prevention of carry-over contamination include the use of suitable and clean equipment. Generally, disposable consumables (tubes, pipette tips, etc.) are highly preferable to reusable equipment. The use of disposable tips containing hydrophobic filters is another very effective measure to minimize cross-contamination. All samples, primer, probes, etc. must be labeled with relevant information such as identity of the content, date of use or preparation, expiration date, concentration, and storage information. Dedicated laboratory coats or disposable lab coats should be available in each room (or section) of the NAT laboratory. Appropriate gloves should be used during all processing steps to prevent sample contamination. The gloves should be changed frequently. Because heat sterilization does not completely destroy DNA, PCR products may lead to detectable contamination of, for example, glass surfaces. Following unique sterilization procedures for different materials such as waste and glass laboratory equipment is advisable.

Carry-Over Prevention with Uracil-*N*-Glycosylase

Contamination by PCR product carry-over can be mitigated by using the commercially available uracil-*N*-glycosylase (UNG) procedure. The procedure involves substituting 2′-deoxyuridine 5′-triphosphate (dUTP) for 2′-deoxythymidine 5′-triphosphate (dTTP) in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification, which can be easily incorporated as a first step into PCR cycling programs. Incorporating dUTP into the amplicon makes the PCR products biochemically distinct from the native DNA template. The enzyme UNG cleaves the deoxyuridine-containing PCR products by opening the deoxyribose ring at the C1 position. When the deoxyuridine-containing DNA is heated during the first thermal cycle, the amplicon DNA chain breaks at the position of the deoxyuridine at the alkaline pH of the PCR reaction mixture and thereby renders the carried-over PCR product nonamplifiable. Thus, any previously generated U-containing amplicon that might have contaminated another sample will become nonamplifiable. As a consequence, false positive results can be avoided. However, it should be noted that UNG has concentration limits above which it does not fully remove PCR carry-over products.

VALIDATION OF NAT SYSTEMS

Assay validation is achieved by

1. ensuring the quality and consistency of assay components, including primers, probes, and enzymes; (including shelf life and contamination control) and
2. establishing the performance characteristics of the NAT assay in terms of reproducibility, accuracy, ruggedness, robustness, specificity, precision, and analytical and clinical sensitivity.

The analytical sensitivity of an assay is defined as the minimum concentration of a reference reagent or standard detected by the test while the clinical sensitivity of a test is determined by testing clinical specimens and determining the 95% LOD. The clinical sensitivity of a test is not necessarily the same as the analytical sensitivity. The closer the reference or standard material is to the samples being tested the closer the correlation.

The principal steps of assay validation are

1. sample preparation;
2. consistent production of critical reagents;
3. use of controls, calibrators, and quantitation standards;
4. specimen and reagent stability;
5. functionality of instruments and software;
6. operator training; and
7. laboratory surveillance for proficiency.

Following assay validation, further QA is necessary to monitor specifications and functional characteristics that have been established by the use of well-characterized reagents of known potency.

Quality Control of Reagents

DNA TEMPLATES

The test specimens used are usually, but not limited to, whole blood, plasma, and serum. Specimen preparation is a key step in the NAT assay and has a major influence on the performance and variability of the assay. Specimen collection is the first step in sample preparation. QC/QA staff should carefully evaluate the effects on the integrity of DNA of collection tubes and temperatures during sample transport. To prevent cross-contamination during specimen collection, aseptic techniques should be used along with closed sampling systems in order to avoid specimen contamination. The use of appropriate sample handling techniques, temperature conditions, and anticoagulants or preservatives should help reduce the risk of contamination. Anticoagulants such as heparin or EDTA may interfere with the NAT assay.

SAMPLE EXTRACTION

The buffers, reagents, and detergent or chaotropic agents used for nucleic acid extraction should be evaluated for inhibitory effects on the NAT assay. Extraction controls, including spiked materials, should be included to monitor the efficiency and reproducibility of the extraction method. Reproducibility of the sample preparation method should be determined under the specimen processing conditions, including sample handling, storage, and shipping conditions. DNA is generally stable, but personnel should take care to avoid storage at refrigerated temperatures for extended periods of time to avoid sample

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degradation. Repeated freeze–thaw cycles can sometimes cause DNA fragmentation. In the case that the target is RNA, it should be noted that RNA is very unstable and specimens should be frozen.

PRIMERS

Primers and probes should be qualified in terms of purity, identity, and functional potency. Purity can be assessed by use of HPLC or mass spectrometry; identity can be established by sequencing; and functionality can be established by the use of reference reagents. However, in many cases, these methods may not be available for in-house testing. In these cases, it may be sufficient to compare lot-to-lot variation of purity and functional potency using relevant methods available in-house coupled with the use of reference reagents.

DNA POLYMERASES

The functionality of enzymes should be determined using reference materials. Enzyme preparations should be tested for other enzymatic activities; for example, exonucleases and DNA- and RNA-dependent polymerase activities and specifications should be established. Lot-to-lot comparison, as well as comparison with the manufacturer's CoA should also be done. Storage conditions recommended by the manufacturer should be strictly followed, and appropriate controls should be used to monitor the stability of enzymes.

Run Controls

The use of controls affords the operator assurance that the assay has performed within accepted specifications. In PCR testing, several steps in the testing process, as outlined above, should be monitored and verified. Multiple controls or controls that serve multiple purposes may be needed for a PCR assay. Controls should reflect the specific technology under development but should typically allow monitoring of ultracentrifugation, extraction, amplification, hybridization, quantitation, contamination, etc. Controls should be similar to the specimen type whenever feasible, although spiked controls may be acceptable.

A negative control is one that does not contain the target sequence or pathogen that is being tested. It should resemble as closely as possible the sample matrix under testing. Multiple negative controls should be examined, including nontarget sequences and nucleic acid-free controls to monitor for false positives resulting from contamination. Because of the high sensitivity of amplification assays, QC/QA personnel highly recommend that sponsors include control measures for the prevention of contamination events.

A positive control is one that contains the target sequence of interest. It should resemble as closely as possible the specimen matrix being tested and should contain an appropriate and defined amount of target sequences. (e.g., kit control).

Specifications for both positive and negative controls should be provided, as well as validation data supporting the proposed assay cut-off/reporting threshold value or the assay's limit of detection. The laboratory should define the source of the controls and calibrators and have a plan for their continued renewal. Controls can be infectious or non-infectious. In the latter case, validation of viral inactivation should be provided.

Reagent controls are often referred to as blanks and could include samples that have no target sequence, no enzyme, no primers, etc. These controls provide additional information about problems encountered in PCR assays.

An internal control is added to each specimen to ensure the overall validity of the individual test results. Internal controls are used to verify sample extraction, amplification, and detection.

External Quality Assessment and Proficiency Testing

Quality assessment of the laboratory is achieved by participation in periodic competency assessment and laboratory proficiency programs. The latter should include the testing of reference reagents and well-characterized panels to measure the technical proficiency of operators. Therefore, care should be taken to prevent cross-contamination, to monitor workflow, and to ensure careful specimen and test sample handling. Evaluation of operator proficiency should include participation in competency and quality assessment programs. Each operator in a particular laboratory should participate in such programs and should demonstrate comparable results.

Data Management

Complete and consistent documentation of all activities performed and all data generated is necessary. Such documentation does not only require the maintenance of records of the data generated through sample testing but also information about reagents and equipment calibration and maintenance. Moreover, any alteration in the assay procedure needs to be introduced through a planned change control process and documented in such a way that change can be assessed by an independent party.

⟨1128⟩ NUCLEIC ACID-BASED TECHNIQUES—MICROARRAY

INTRODUCTION

Microarrays are microscopic spots of DNA (measured in micrometers) arranged in an ordered manner (columns and rows) on a planar surface so that each DNA spot can be uniquely identified to facilitate an accurate analysis of the data. The DNA

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spots, also called array elements, are specific DNA molecules of known or unknown sequences and can be of similar or different nucleotide lengths. Samples of these mixtures are placed in fixed locations on the microarray.

Unlike conventional probes, which are a specific DNA or RNA sequence labeled with radioactive, fluorescent, or chemiluminescent tags (see *Nucleic Acid-Based Techniques—General* ⟨1125⟩, *Glossary*), the array elements are referred to as probes when the sequence information of the array elements is known, despite not being labeled. In this context, the target refers to labeled nucleic acids in solutions that are hybridized to the array elements or probes. The purpose of a microarray experiment is to identify the sequence of these labeled nucleic acids and/or determine their content. Compendial applications at this time are limited but may increase with wider use of microarrays in diagnostics and in drug discovery, development, registration, and control applications. When used for compendial purposes, standard assay development and validation approaches with availability of suitable reference materials are likely to apply.

Microarrays can range from hundreds to thousands of array elements (low density), tens to hundreds of thousands of array elements (high density), to millions of array elements (very high density). In addition to the use of planar surfaces for microarrays, the array elements can also be immobilized on individual support particles, such as beads. In these cases the array elements are identified by the particles themselves rather than specific locations on an array. The advantages of using microscopic spots on the array include high density, fast hybridization kinetics, and low sample volumes. Microarrays greatly speed up the acquisition of data, and in some cases increase the predictive power of results, by comparison with conventional nucleic acid-based assays. This is achieved by miniaturization, multiplexing, and parallel execution of nucleic acid-based tests that traditionally are performed in tubes, plates, or capillaries as described in general chapter ⟨1125⟩ (see also *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩).

The principle of microarray analysis is the specific binding of the target DNA molecules to the probes or array elements. The ordered array of rows and columns of spots allows highly automated detection and analysis. DNA microarrays are manufactured, processed, detected, and analyzed in a number of different ways and have many applications. With the aid of computers, laboratory automation, and high-resolution detection devices, microarrays produce large amounts of data and are the analytical tool of choice to unravel the molecular complexity of DNA or expressed RNA.

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are described in chapter ⟨1127⟩. The present chapter covers the general field of microarrays, but detailed treatment of various application-specific microarrays, including data analysis and validation, are excluded from this chapter at this time. The following sections address the major applications of microarrays, sample processing, labeling, workflow, detection, and analysis of data. Several of these sections, for example, sample preparation and labeling, overlap with chapters ⟨1126⟩ and ⟨1127⟩, and cross references are made accordingly. Finally, regulatory aspects of microarrays will be discussed.

GENERAL PRINCIPLES OF MICROARRAY EXPERIMENTS

Types and Applications

Microarrays are most widely used in three types of analysis: gene expression, microarray-based comparative genomic hybridization (or array comparative genome hybridization, aCGH), and single nucleotide polymorphism (SNP). In brief, gene expression microarrays generally measure messenger RNA in a cell; aCGH analyzes DNA copy number variations, chromosomal additions, and deletions in genomic DNA; and SNP microarrays are used in genotyping to analyze single nucleotide polymorphisms (see ⟨1129⟩). Within each type of microarray, various platforms, both manual and with various levels of automation, are available. *Table 1* summarizes the three major types and most common applications, as well as the target for each application, the probe, and the complementary nucleic acid techniques (see ⟨1126⟩, ⟨1127⟩, and ⟨1129⟩).

Table 1. Major Types and Applications of Microarrays

Types	Application	Target	Probe	Complementary Technology
Gene Expression	Gene Expression	mRNA	Oligonucleotide/cDNA	qRT/PCR, Northern Blotting
aCGH	aCGH CNV	DNA	Oligonucleotide/ cDNA/Pac, Yac, Bac	Cytogenetic chromosome analysis
SNP	SNP Genotyping	DNA	Oligonucleotides	Sequencing
	SNP	Amplicons	Oligonucleotide	Sequencing
	SNP	Oligonucleotide	Amplicon	Sequencing

GENE EXPRESSION MICROARRAYS

Gene expression microarrays are used to measure the relative level at which a certain gene is expressed. They are a powerful tool for target gene discovery, molecular tumor characterization, diagnosis, classification, treatment, and monitoring of diseases. Underlying molecular subgroups that are active in diseases have been identified by observing distinct and recurring gene expression subsets found within diseased tissues. Gene expression microarrays are also used to measure changes in gene expression over a given period of time, e.g., within various stages of a cell cycle or by identification of gene mutation(s) that lead to cancerous growth. Another application for gene expression microarrays is the development of new drugs, e.g. by measuring the down-regulation of a gene associated with a particular disease to monitor the effectiveness of a new drug. When the expression levels from a set of genes are measured, the term gene expression signature (biomarker or classifier) is used.

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Other examples of biomarkers or classifiers are drug activity classifiers that are used to diagnose the mechanism of action of a drug or toxicity classifiers that are used to diagnose and develop dosage parameters for a patient.

aCGH Microarrays

In contrast to gene expression microarrays, aCGH microarrays target segments of DNA rather than individual genes (this is, similar to chromosomal banding and traditional comparative genomic hybridization). In an aCGH microarray, the array elements, which are large pieces of genomic DNA or specially designed oligonucleotides, are used to identify a known chromosomal location or changes. The primary advantage of aCGH is the ability to detect DNA copy changes at multiple loci in a single assay and to do so at a much greater resolution compared to traditional CGH. Depending on their design, aCGH microarrays provide distinct advantages over conventional cytogenetic analysis such as karyotyping and fluorescence in situ hybridization (FISH) because they have the potential to detect the majority of microscopic and submicroscopic chromosomal abnormalities. Compared to aCGH, these conventional cytogenetic techniques have low throughput, are labor-intensive, and often require specially trained staff to perform tests in a consistent manner. aCGH microarrays are also useful for the detection of cancer by monitoring the loci of oncogenes and tumor suppressor genes.

SNP Microarrays

SNP microarrays identify the presence of known sequence polymorphisms by analysis of the pattern of hybridization to a series of probes that are specifically complementary either to wild-type or mutant sequences. If the SNP or set of SNPs associated with a particular disease are known, SNP microarrays can be used to identify a disease in an individual. SNP microarrays provide an efficient and inexpensive tool for simultaneously studying multiple genetic variations in multiple samples.

Design of Microarrays

The following sections discuss the design of the three types of microarrays described above and the suitability of the materials used for the microarray probes for each of the three types.

Gene Expression Microarray

These microarrays are the most common type of microarray in use today. The array elements consist of either cDNA derived from mRNA of known genes but of unknown sequence, or oligonucleotides for which detailed sequence information is available. Oligonucleotides are preferred array elements because of the affordable cost of synthesis and the large amount of sequence information now available for specific genes or gene fragments. These can be arrayed in specific patterns to enable accurate analysis of related gene sequences and gene families in a single hybridization assay. The following general principles apply to oligonucleotide design for gene expression microarrays:

1. Oligonucleotides should be 25–70 mers.
2. Oligonucleotides should include appropriate controls (i.e., oligonucleotides corresponding to sequences from a different organism).
3. All oligonucleotides should map to within 1000 nucleotides of the 3′ end of cDNAs and should correspond to the coding strand.
4. Sequence repeats, stretches of polyA, G, C, and T and extremes of T_ms should be avoided.
5. Oligonucleotides should be compared to sequences in existing databases to avoid cross-reactivity (less than 70% sequence identity with nontarget sequences is preferable).

In addition to oligonucleotides, PCR amplicons and double-stranded DNA (dsDNA) are also used as probes. However, the PCR amplicons require purification to remove enzymes, salts, nucleotides, and other contaminants from the amplification process that could interfere with the binding of the probes and could also inhibit hybridization. In addition, the preparation of dsDNA probes for spotting is labor intensive and expensive. Moreover, dsDNA probes can have repetitive sequences that compromise hybridization specificity. When sequence information is unavailable, dsDNA remain the probes of choice because unknown dsDNA probes can still be used to study gene expression.

aCGH Microarrays

These microarrays traditionally use bacterial artificial chromosomes (BACs) of 100–200 kilo-base pairs per DNA segment as the array elements. However, the large-scale DNA isolations or PCR amplifications of such large-insert clones are elaborate and time consuming. As is the case in expression profiling applications, aCGH microarrays have transitioned from dsDNA targets to oligonucleotide targets. Oligonucleotide libraries or ready-made microarrays can now be purchased, saving considerable time and effort.

SNP Microarrays

Depending on the application, SNP microarrays can use both amplicons and oligonucleotides as probes. In one of the most common formats to detect mutations in a gene sequence, the probe is that of a single gene in which the sequence differs by a single nucleotide polymorphism from the sequence of the other probes for that gene in the same microarray. For the discrimination of only one mismatch, short oligonucleotide probes (15–30 bp) maximize the destabilization caused by mispairing and are therefore used for the detection of SNPs.

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Manufacturing of Microarrays

Microarray elements are deposited onto a solid support, the most widely used of which is glass. Microarray manufacturing can be divided into two main categories, direct synthesis of the probes on the microarray (in situ) or synthesis of the probes before spotting on the microarray (ex situ). In situ synthesis is generally used for higher density microarrays but is limited to nucleotides of approximately 25–100 bases. With increasing nucleotide length, the likelihood of truncated products increases because of the limited stability of building oligonucleotides in situ. In contrast, ex situ microarray manufacturing can put any premade material into a microarray format, including oligonucleotides, PCR products (amplicons), complementary DNA (cDNAs), and BACs.

The main techniques for in situ synthesis are photolithography, maskless lithography, and ink jetting. Microarrays are generally manufactured commercially, although for a small number of low-density microarrays, the end user can manufacture the microarrays using a low-throughput microarray manufacturing robotic instrument (a personal microarrayer). However, only maskless lithography and ink jetting are available for end user manufacturing. In photolithography, a glass substrate containing a photomask, which is chemically prepared so that particular nucleotides bind to specific positions, is used to synthesize the oligonucleotides on the substrate. The masks predetermine which of the nucleotides are activated when flooded with one of the four types of nucleotides. The process is repeated until the required number of bases is synthesized. The manufacture of these microarrays uses computer algorithms and multiple spots to cover the gene of interest. Maskless lithography uses a digital micromirror device that uses a solid-state array of miniature aluminum mirrors to create virtual masks that replace the physical photomasks. A computer controls the desired pattern of UV light via individual mirrors. Each digital micromirror in turn controls the pattern of UV light projected onto the glass in the reaction chamber, which is coupled to a DNA synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next nucleotide will be coupled. The patterns are coordinated with the DNA synthesis chemistry in a parallel, combinatorial manner so that hundreds of thousands of unique oligonucleotides can be synthesized in a single microarray. Ink jetting is accomplished by building up the nucleotides, base-by-base, in repetitive print layers using standard phosphoramidite chemistry. Inkjet heads similar to those used in commercial inkjet printers are connected to bottles that contain the four different phosphoramidite nucleotides that make up the building blocks of in situ nucleic acid synthesis. The advantages of inkjetting and maskless lithography are flexibility in design and the ability to make small batches of arrays quickly.

The two main types of ex situ manufacturing techniques are microspotting pins (contact printing) and piezoelectric printing (noncontact). The technology excels at printing multiple probes many times over numerous surfaces with one small-volume loading of probe. Spot size and delivery volume are controlled by the size of the end of the tip, and many tip sizes are available. A piezoelectric printing mechanism uses a small dielectric crystal in contact with a glass capillary that holds the sample fluid. Application of the voltage results in ejection of fluid from the tip, resulting in drop volumes from hundreds of picoliters to several microliters.

General Experimental Considerations

Regardless of the type and application, all microarray experiments have a similar workflow: amplification step, labeling, hybridization, and wash steps, followed by scanning, quantitation, and reporting. The experimental design determines the type of microarray used, number of spots required, and the specific sets of nucleic acids on the microarray. The experimental design also influences the platform used, such as the number of spots, surface type, nucleic acid type, throughput, resolution, and number of colors that can be detected in a single assay. Platforms can be open (support is available from multiple vendors) or closed (support from a single vendor). In general, experimental designs that require a high density of spots and quantitation are more difficult and expensive to implement than qualitative assays.

Microarray Sample Considerations

Sample extraction, isolation, and preparation should be carefully chosen in order not to alter the ability of the resulting target to hybridize to the microarray. In general, sample preparation issues are the same for microarrays as for other laboratory techniques such as qPCR (quantitative PCR) and sequencing (described in chapters ⟨1126⟩ and ⟨1127⟩). RNA, cDNA, genomic DNA, and PCR products are some of the sample types analyzed with microarrays. In some genotyping applications, specific alleles are used both as array elements and targets.

As with any nucleic acid technique, the quality of the nucleic acid is critical for the microarray experiment. The nucleic acid should be pure, intact, and accurately quantitated before use ⟨1126⟩. In particular, the presence of contaminating DNA in total RNA samples may cause problems in microarray analysis because some labeling methods label both RNA and DNA with equal efficiency. For some applications in which even trace contaminants with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. For example, contaminating, labeled DNA can hybridize with microarray targets leading to high-level hybridization signals that are not derived from RNA transcripts, thus resulting in an inaccurate estimation of the target RNA concentration because both nucleic acid species are quantitated at the same wavelength.

A major consideration in any microarray experiment is the availability of adequate amounts of sample nucleic acid for analysis. For example, sample from laser-capture microdissection, needle tissue biopsies, or other small clinical samples do not yield sufficient RNA (for expression microarrays) or DNA (for aCGH microarrays) and must be amplified before analysis. It is critical that the amplification procedures for amplification of mRNA be so designed that the final mixture of amplicons accurately reflect the distribution of mRNA species in the sample. Uniform amplification of genomic DNA for aCGH microarrays can be achieved by the use of multiple displacement amplification (MDA), which overcomes the nonuniform amplification of genomic DNA that occurs in PCR-based amplification methods that use degenerate oligonucleotide primed PCR (DOP-PCR). For SNP arrays where specific alleles are the target of interest, nonuniform amplification is not an issue, and samples can be amplified (and labeled) by PCR, multiplex PCR, and WGA (see ⟨1127⟩).

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Microarray Labeling

The targets for a microarray are a population of nucleic acids that are extracted from a sample and are appropriately labeled. Many methods can be used for labeling targets (see ⟨1127⟩), but fluorescent labeling is the most widely used because it offers high sensitivity and a superior dynamic range. An added advantage is the ability to detect two or more signals in a single experiment. The method of labeling depends on the microarray type. The two methods used to fluorescently label targets for gene expression microarrays, direct and indirect labeling, have been described in ⟨1127⟩. In general, the second method (indirect labeling), in which the label is added via a linker, requires less starting material and is less expensive. Published reports have shown that this method yields results similar to those obtained from directly labeled samples. In microarray aCGH, a patient's DNA and reference DNA (300–1000 ng) are typically fluorescently labeled with red and green fluorescent dyes, respectively, often using a random priming protocol. Random prime labeling uses a high concentration of Klenow enzyme whereby genomic DNA is digested with restriction enzymes and hybridized with random primers. The primers are extended by the 5′–3′ polymerase activity of Klenow, resulting in a strand displacement activity with the direct incorporation of labeled nucleotides. SNP microarrays using oligonucleotides as array elements are labeled using fluorescently labeled nucleotides in both single and multiplexed PCR reactions, followed by a purification step to remove unincorporated dyes. Where amplicons are used as array element, labeled oligonucleotide probes are synthesized using phosphoramidite chemistry.

Hybridization and Wash

Hybridization should be carried out under conditions that minimize annealing of noncomplementary fragments. The wash steps following a hybridization reaction are optimized to provide the highest possible specificity, signal-to-noise ratio, and reproducibility (see ⟨1126⟩). Before hybridization, double-stranded probes and targets should be denatured, and nonspecific sites should be blocked. Microarray surface chemistries are designed to capture all nucleic acids with high efficiency, so the free-binding groups on the surface must be blocked or inactivated to prevent nonspecific binding of labeled material that could compromise the signal-to-noise ratio. Surfaces are blocked and washed with various aqueous-based buffers that typically include salts, detergents, and blocking agents such as low molecular weight, hydrolyzed proteins. The purpose of the posthybridization washes is to remove all unattached and nonspecifically bound label from the surface and probes. In general, both automated and manual washes are done in saline sodium citrate/sodium dodecyl sulfate (SSC/SDS) buffers of various concentrations and at different elevated temperatures depending on the stringency required. After the final wash step, microarrays using fluorescent targets are dried immediately by centrifugation or in a nitrogen stream. Hybridized microarrays must be stored in the dark and should be scanned as soon as possible. Some fluorescent dyes used in microarray analysis are subject to degradation by environmental ozone, and in these cases ozone levels in the experimental environment must be less than 5 parts per billion. Specialized ozone-free hoods are made to protect microarray dyes.

Microarray Detection

Regardless of the microarray type, each spot on a microarray represents a unique probe sequence to which a single, labeled target is bound, and this specific binding allows detection and quantitation of the target. This is achieved by the emission of light (photons) at a particular wavelength by the fluorescently labeled duplexes when the microarray is exposed to light of specific wavelength from an excitation source. The emitted fluorescent light is converted to electrical energy by a detector. The detector is either a photomultiplier tube (PMT) or a charge-coupled device (CCD) with specially designed optical paths that collect the raw data from microarrays (scanning). The detector filters and optical paths are designed to detect specific fluorescent dyes at sufficient resolution while eliminating crosstalk when two or more dyes are used on a single microarray. The resulting signal is proportional to the number of photons emitted by the microarray. These signals are used to create a digitized image showing the presence and quantitation of specific targets.

Samples can be scanned from a single wavelength channel or can be sequentially scanned from two channels. For instance, for a single-channel microarray platform, a sample is typically labeled with a fluorophore that emits a signal in the red channel. For a dual-channel microarray format, a second sample can be labeled with a dye that emits in the green channel. Dual labeling is used in some experimental designs, such as expression microarrays, to measure the overexpression of a gene associated with a disease state. In such experiments, cDNAs derived from the mRNA of normal and diseased tissues are differentially labeled, mixed, and tested on the same slide in a competitive hybridization reaction. The resulting ratios of the two colors reflect the relative abundance of the labeled material within each sample. Similarly, calculating the fluorescent ratios from each target on an aCGH microarray allows the mapping of gains and losses for a chromosome of interest.

Microarray Image Processing

Most microarray scanners detect and acquire one, two, or more colors (via one, two, or more channels). The optical path of the system minimizes overlap between the spectra (crosstalk) and allows acquisition of two spectrally separate images. In many cases, the images are represented as a red and a green image. When two colors are used, the ratio of the two fluorescence images eliminates artifacts caused by regional bias and irregular spot size. When one color is used, the fluorescence signals from two or more microarrays are normalized and can be compared with each other. Diameters of spots printed on the arrays range from 10 µm to just under 1000 µm, and the resolution of scanners ranges from 1 to 50 µm. Thus, depending on scanner resolution, variable amounts of pixel data can be collected per scan over an entire microarray.

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MICROARRAY IMAGE ANALYSIS

The analysis of scanned images usually involves three tasks: spot finding or gridding, image segmentation, and spot quantification.

Spots are initially assigned specific coordinates, and the process of spot finding or gridding can range from manual to fully automatic, depending on the image-processing software used. This takes into account the individual size and shape of each spot and adjusts for uneven rows and columns that may be produced by the printing process.

The process of segmentation partitions the entire image to foreground or background pixels and relies on the spatial and intensity properties of each pixel. There are four main types of signal segmentation that have been used for spotted arrays. The simplest method is spatial segmentation which places two circles (inner and outer circles) of fixed but different sizes over each spot to demarcate probe signal from the immediate background signal. On the one hand, because of the irregularity of spot sizes on some microarrays, the actual area inner circle may be larger than the diameter of a spot and thus will contain background pixels. On the other hand, artifacts and signal can be found in the area between the inner and outer circles and contribute to the background signal. The second method, intensity-based segmentation, distinguishes signal pixels from background pixels based on the spot intensities within a target region. In this case, a certain percentage of pixels within the top-ranked intensities may be classified as signal pixels. The advantages of this method are simplicity and speed, but the drawback is the inability to distinguish between artifacts and signal and the tendency to detect low signals that are close to background. The third method is a statistical approach known as Mann-Whitney segmentation that combines information from spatial and intensity-based analysis. Here, background pixels located outside the inner circle set are used to determine a threshold intensity level for a signal within the inner circle. The limitation of this method is that a large amount of spot irregularities and artifacts can reduce its accuracy. The fourth method, the trimmed measurement segmentation method, also combines spatial and intensity information and measures signal distributions inside and outside the inner circle. The method trims the upper and lower extremes of each distribution to allow removal of signal from artifacts and incorrectly located background or foreground signal pixels.

The main assumption of spot quantification is that the total fluorescent intensity from a spot is proportional to the expression level of the labeled transcript. This is highly dependent on a number of factors, including target preparation, hybridization conditions, and signal detection within the linear dynamic range. If the amount of probe deposited during the microarray manufacturing procedure varies from spot to spot and from array to array, thus resulting in different sized spots, the sum or total signal intensity can be variable and inaccurate. To correct for this variation, microarrays should be spotted via homogenous surface chemistry that has a fixed binding capacity. This ensures the same amount of probe at each spot location. Alternatively, spots can be quantified by taking the mean, median, or mode of intensities of all signal pixels determined to be foreground signal. The more robust methods that protect against outlier signals are the trimmed mean (where a certain percentage of top and bottom signals are trimmed before calculation of the mean) and median signal intensities. When two different fluorophores are used, the intensity ratio can be used to correct for variable probe amounts and can be calculated from mean, median, and mode intensities from each channel.

MICROARRAY DATA ANALYSIS

Particularly dense formats of microarrays that contain tens of thousands to millions of probes per chip or slide generate a large volume of raw data per array, which requires the use of specialized data-analysis software. Microarray software programs are designed to extract primary data, normalize the data to remove the influence of experimental variation, and link probes to relevant gene and sequence-derived targets. Software programs are also available to apply statistical methods, analyze, visually display, and manage data in order to extract biologically meaningful information. The major parts of data analysis are normalization, background correction, and ratio calculation.

Normalization systematically adjusts microarray raw data in an effort to reduce the variability brought about by differences in the manufacture and processing of the microarrays and by technical variables so that true biological differences between samples can be detected. The wide range of normalization methods precludes a detailed discussion of the topic in this chapter, and currently there are no standards for normalization. Commonly used algorithms are selected based on the microarray type, the number of fluorophores used, and the samples being studied. Some methods are built into the manufacturer's software, but others are available from commercial sources or open-source software providers.

Background correction eliminates low levels of noise in microarrays stemming from both the inherent noise of the detection instruments and from the surface chemistry used in manufacturing. Several contaminants acquired from microarray processing can cause high levels of background that must be corrected before data analysis.

In two-color microarrays, the ratio of signal intensities of array elements of two co-hybridized samples is used as a relative measure of gene expression. In single-channel systems, the ratio can be calculated between signals taken from two different samples (one sample is a reference sample) hybridized on individual microarrays. Thus, the resulting data from microarrays does not represent an absolute quantification but rather a relative level of RNA or DNA against a reference sample or control.

Quality Control and Quality Assurance

As with any diagnostic assay, quality control and quality assurance are critically important. Microarrays must demonstrate robustness and reproducibility. The general quality control and assurance steps outlined in chapter ⟨1127⟩ for nucleic acids and NAT also apply to microarrays. Unlike other diagnostic tests, no reference reagents are available at present for quality control of microarrays, and regulatory guidance is emerging. FDA has issued a draft guideline titled "In Vitro Diagnostic Multivariate Index Assays." This guidance addresses the definition and regulatory status of a class of in vitro diagnostic devices referred to as in vitro diagnostic multivariate index assays (IVDMIAs), and microarrays fall into this category. The guidance addresses premarket pathways and postmarket requirements with respect to IVDMIAs.

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Several unintended sources of variability that are specific to microarrays can extensively affect signal intensities and the accurate derivation of a true signal that accurately reflects the labeled transcript. A major source of variability is spot quality. Measurements of spot quality at the processing stage permit removal of spots with poor or questionable quality. Other sources of variability are artifacts, for example, regional shifts (rise or fall) in an array's overall signal that can be visualized within single chips or in-composite data derived from multiple chips. These changes can be distinguished from actual variability because they are nonrandom, and patterns can be detected by visualizing signals over the entire area of the chip. When dyes of different spectral properties are used to label two different samples in a competitive hybridization reaction using a single array, differences may arise because of labeling bias rather than gene expression level. For instance, the green channel may appear consistently brighter than the red channel despite the fact that there are no real differences in expression. Hybridization with reverse dyes can ensure detection and elimination of dye bias effects. As with any quantitative assay for RNA, the integrity of the sample affects its measurement, and sample quality is an important determinant for accuracy. For instance, because labeling is directed from the 3′ to the 5′ end but RNA degrades from the 5′ end, degraded RNA leads to high 3′/5′ ratios, resulting in nonuniform labeling across the entire transcript. Finally, variability can be introduced during the processing of microarrays, which is a relatively complex procedure that involves multiple steps such as labeling, hybridization, washing, and staining (technical variables). Such variability can mask true differences in the samples tested.

When used as a diagnostic test, the microarray should demonstrate robustness, reproducibility, a high degree of correlation to the original format, and reliable prognosis prediction. The microarray ideally should contain at least 2–3 replicate spots for each reporter gene to ensure intra-assay reproducibility. With a two-channel microarray, a reference sample pool can be hybridized in the complimentary fluorescent channel so that data can be expressed as log ratios, which reduces the need for extensive normalization. Interassay reproducibility of test results and stability over time can be tracked by using a number of reference samples that, when labeled and hybridized, represent a spectrum of predictive endpoints (for instance, high risk, borderline risk, low risk) and that should fall within a predetermined range of results. Failure of these controls should result in rejection of results of samples in the same assay run. If the assay is performed at many sites, site-to-site reproducibility is imperative and must be assessed. The reproducibility of the assay with regard to tissue extraction also must be determined, and the quality of tissue specimens or RNA should be specified clearly (for instance, percentage of tumor cells within a specimen).

In conclusion, microarray experiments should be carefully designed and conducted in order to minimize variability and to yield data that accurately relate to the samples analyzed. In addition, biomarkers of interest should be analyzed and verified using an alternative platform such as qRT-PCR that should be shown to be reproducibly detected in the same and different samples. The development of reference standards, especially when microarrays are used as diagnostic tests, is the next step to ensuring the quality and validation of microarray results. With the shift from custom-built to commercial microarrays, issues with reproducibility, standardization, and quality control have been largely addressed by the stringent quality controls used in commercial manufacturing.

⟨1129⟩ NUCLEIC ACID-BASED TECHNIQUES—GENOTYPING

INTRODUCTION

This chapter outlines techniques for detecting single-base DNA differences and other types of polymorphic DNA sequences that occur in the three billion bases that make up the human genome. The most common genetic variation is a single nucleotide polymorphism (SNP), which is a simple change in one base of the gene sequence. SNPs occur on average every 1000 bases and account for a significant amount of inter-individual variability. SNPs can predispose individuals to disease or influence their response to a drug. Approximately 1.8 million human SNP loci have been identified, and more are likely to be discovered in the coming years.[1]

Common approaches for detecting SNPs and other types of polymorphic DNA sequences are described in the following sections. These approaches encompass a variety of techniques, such as nucleic acid amplification techniques (NAT), real-time NAT, and microarrays, the principles of which are covered in more detail in related chapters. This chapter focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences.

SNP GENOTYPING TECHNOLOGIES

Although the usefulness of studying SNPs for gene mapping and disease association studies is apparent, a single standardized procedure for SNP genotyping has not been adopted. Various approaches for performing SNP genotyping have been developed to meet a wide range of needs, including throughput capacity, ease of assay design, accuracy, and reliability. Available procedures can also be divided according to whether they are based on identifying known SNPs or whether they can be used to screen for unknown SNPs. To identify the most appropriate SNP genotyping procedure for a specific application, the throughput requirements in terms of the number of SNPs to be analyzed per sample (multiplexing level) and the sample throughput need to be determined because different approaches may work best depending on these requirements.

Most procedures used for genotyping SNPs depend on polymerase chain reaction (PCR) amplification of the genomic regions that span the SNPs followed by the actual genotyping reaction. PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions limits the throughput of many of the current SNP genotyping assays. The following sections outline several of the major approaches currently in use for SNP genotyping. In many cases the underlying technology can be modified to meet the specific application requirements in terms of sample throughput and number of SNPs detected. In general,

[1] Database of Single Nucleotide Polymorphism (db SNP) Build 128 is available from National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/projects/SNP/index.html.

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real-time PCR-based procedures are better suited to higher sample numbers, and array-based procedures are better suited to the simultaneous detection of many SNPs. Newer technologies based on multiplexed array formats are also emerging and will be suitable for high sample numbers and many SNP applications.

Sequencing

Sequencing is the definitive procedure for DNA analysis, and its use for SNP detection allows unambiguous identification of base changes (see *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩ for nucleic acid sequencing). The standard technology is expensive, and the procedure is time consuming and labor intensive and suffers from low sample throughput. Sequencing is a useful confirmatory tool, and it has applications in situations when other technologies are not appropriate, but is not the most cost-effective solution for the majority of SNP genotyping applications that require the identification of only one or a few bases.

Restriction Fragment Length Polymorphism Analysis

The first widely used procedure for the detection of polymorphisms exploited alterations in restriction enzyme sites caused by SNPs, leading to the gain or loss of cutting events. PCR–restriction fragment length polymorphism (RFLP) analysis comprises PCR amplification of a fragment of interest and subsequent digestion with a restriction enzyme. The fragments produced are typically analyzed by a size fractionation procedure, usually gel electrophoresis. Because of its simplicity, the procedure has been and still is extensively used, although it entails certain limitations: only a subset of polymorphisms that reside in an endonuclease restriction site can be studied with the conventional procedure; incomplete digestion due to suboptimal processing can produce misleading digestion patterns; and the procedure is less amenable to automation than are other SNP genotyping procedures.

Probe Hybridization

The basis of many SNP genotyping procedures are DNA hybridizations that make use of the stronger binding of a DNA probe to a perfectly matched complementary target than to a target that contains a single base mismatch. The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in the late 1970s and subsequently was used to detect the sickle-cell mutation in the beta-globin gene by Southern blot hybridization. The invention of PCR facilitated the further development of probe-based assays for genotyping SNPs in complex genomes.

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions but also by the secondary structure of the target sequence and the nucleotide sequence flanking the SNP. Therefore, prediction a priori of the reaction conditions or the sequence of the ASO probe that will allow optimal discrimination between two alleles using ASO hybridization is difficult. These parameters should be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an extremely difficult task.

One approach to counter the problem of assay design is to carry out multiplex ASO hybridization reactions on arrays that carry multiple probes for each SNP that will be analyzed. This involves using probe sets in which the SNP occurs at different positions along the probes. It becomes feasible to include large numbers of ASO probes per SNP when one uses high-density arrays that can carry as many as 10^6 probes per cm^2.

Another approach is to use base analogues such as locked nucleic acid (LNA), which is described in detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. For applications that involve few SNPs but many samples, homogeneous real-time PCR approaches have been developed. These include the use of fluorescent probe chemistries such as hydrolysis probes, stem-loop probes, and FRET (fluorescence resonance energy transfer) hybridization probes. The principle of these assays is discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. For SNP detection, the basis of many assays is the selective binding of the ASO probe to its perfectly matched target sequence, resulting in energy transfer and generation of a fluorescence signal. Probes designed with specific secondary structures tend to form a stem–loop structure that destabilizes mismatched hybrids, increasing their power of allele distinction as compared with that of linear ASO probes. Hydrolysis probes modified with minor groove-binder molecules that increase target affinity show improved powers of allele discrimination. The use of two probes, each labeled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. Limited multiplexing can be achieved by using probes labeled with different fluorophores. In the fluorescent probe–based assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtiter plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR. In this case the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence, and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results.

A third approach involves heating the reaction after PCR has been completed in order to disassociate the probe from the target. Each duplex has its own specific T_m, which is defined as the temperature at which 50% of the DNA becomes single stranded. The T_m depends on the stability of the probe–target duplex. Perfectly matched probe–target duplexes have a greater stability and hence a higher T_m than does the same duplex containing a single base mismatch. By continuously monitoring the fluorescence during the heating phase, analysts generate a "melt curve" that measures the changes in fluorescence that result when the probe denatures, or "melts," away from the amplicon. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

Because no post-PCR processing or label-separation steps are required, homogeneous real-time PCR assays are simple to perform, making them useful for high-throughput genotyping applications. The optimal probes must be designed individually for each SNP, and the assays are therefore most efficient when a limited number of SNPs is analyzed. The cost of probes modified with fluorescent and quenching moieties may also be a limiting factor in the high-throughput application of the assays.

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Primer Extension

In this technique, an oligonucleotide is used to prime DNA synthesis by a polymerase enzyme, as performed in a standard PCR or sequencing reaction. Variations of the technique exist. Allele-specific PCR uses two primers, each fully complementary to one of the SNP alleles, with the SNP position being at the 3′ end of the primer, and with a common reverse PCR primer to selectively amplify the SNP alleles. Because only perfectly matched oligonucleotides will prime DNA polymerase extension, product will be detected only from the reaction containing the perfectly matched primer.

Agarose gel electrophoresis is used to detect the amplified products, although homogeneous, real-time, allele-specific PCR approaches have also been developed using primers labeled with different fluorophores or a fluorescent dye that intercalates with the double-stranded PCR products or by performing amplicon detection using probes such as hydrolysis and hairpin (stem–loop) probes. When using intercalating dyes or labeled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, one may find that the specificity of the procedure may be compromised owing to primer–dimers and other spurious amplification products that will not be distinguished from the actual PCR product. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the hydrolysis and hairpin probe assays, the homogeneous allele-specific PCR procedures are best suited for the analysis of a limited number of SNPs in large sample collections. Array-based approaches for greater SNP multiplexing have also been developed.

In procedures based on single nucleotide primer extension (sometimes known as minisequencing), allele discrimination is based on the high accuracy of nucleotide incorporation by DNA polymerase. A primer is used, and its 3′ end is positioned on the base just preceding the SNP to be tested. The DNA polymerase is then used to incorporate labeled ddNTPs, each labeled with different fluorescent dyes. After the labeled oligonucleotides are separated from the nonincorporated ddNTPs, the results can be scored on a fluorescence plate reader. In addition to fluorescent tags, ddNTPs may be labeled with biotin or haptens and then detected indirectly through antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminiscent markers in ELISA formats.

Multiplexing of this procedure has also been described to reduce costs and improve throughput. In these procedures, the different loci genotyped simultaneously are separated either by gel electrophoresis or by hybridization to arrayed tags. Primer extension directly on a solid support such as a microarray is also possible. The immobilization of the single-stranded primers on the solid support may be through biotin–avidin–streptavidin reaction or covalently via 5′ disulfide groups.

Mass spectrometry using techniques such as matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used to determine the identity of the ddNTP incorporated based on mass. A difficulty with MALDI-TOF MS is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. Such enzyme-assisted procedures have proven to be more robust and to provide more specific allele discrimination than does ASO hybridization at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization is minimized.

Ligation

In the oligonucleotide ligation assay (OLA), oligonucleotides are designed so that they meet at the position of the SNP to be tested. Enzymatic joining, using a DNA ligase, occurs only when the match is perfect. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of the SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microplate wells by colorimetric approaches. Multiplexing and the use of gel separation have also been described.

OLA has also been used in microarray formats with one of the ligation probes immobilized or with immobilized single stem–loop probes. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays or on microparticles that carry a generic set of oligonucleotides that are complementary to a "tag" sequence on one of the ligation probes. In practice, thermostable ligases are frequently used for genotyping SNPs in combination with PCR before allele-specific ligase detection reactions. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET. Compared with DNA-polymerase-assisted primer extension procedures, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides, which increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA). RCA can be used to amplify the ligated circularized padlock probes to a level required for detecting single-copy sequences. A homogeneous, isothermal assay for genotyping individual SNPs in a microtiter plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labeled hairpin primers.

Displacement

The invader assay uses the property of flap endonucleases (FENs) for removing redundant portions (flap) from the 5′ end of a downstream DNA fragment overlapping an upstream (invader) DNA fragment. An invader oligonucleotide is designed with its 3′ end on the SNP to be tested. Two oligonucleotide signal probes are also designed, overlapping the polymorphic site and each corresponding to one of the alleles. After displacement of the signal probes by the invader probe, FEN-mediated cleavage occurs only for the perfectly matched allele-specific signal probe. Generation of the cleaved fragment is monitored by using it in a second reaction as an invader probe to cleave a FRET probe. This assay does not require PCR amplification of the locus to be tested, and scoring can be done using a simple fluorescence plate reader.

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Pyrosequencing

In the pyrosequencing procedure, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate (PPi), which is released on incorporation of deoxynucleotide triphosphates. The genotype of an SNP is deduced by sequential addition and degradation of the four nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtiter plate format. Using pyrosequencing, the apparatus can determine short 30 to 50 bp sequences of DNA that flank an SNP. A limitation of the procedure is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes. An advantage of the procedure is that any new polymorphism will be detected. However, specific equipment is needed for the injection of the nucleotides.

Single-Strand Conformation Polymorphism and Heteroduplex Analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis were among the first procedures established for the detection of SNPs. Conventional SSCP analysis involves denaturing PCR-amplified fragments and subsequent formation of sequence-specific secondary and tertiary structures of the single strands during nondenaturing gel electrophoresis. The electrophoretic mobility then depends on the 3-D shape of the single-stranded molecules. One single base difference in DNA fragments of up to 300 bp will usually change the conformation in a way that can be detected by nondenaturing PAGE.

The traditional polyacrylamide gels and ^{32}P-labeled fragments are frequently being replaced by fluorescently labeled fragments and automated capillary electrophoresis. The simplicity of the procedure, combined with automation and short analysis time, contribute to high-throughput analysis at relatively low cost. If the denatured PCR products are allowed to slowly re-nature, they form DNA duplexes. The duplexes with the same sequence on both strands (homoduplexes) or with a single base pair mismatch on one strand (heteroduplexes) have different electrophoretic mobility in a native gel. In the case of a single base pair substitution, the heteroduplex can easily be separated from a homoduplex.

In other versions of the technique, denaturing high-performance liquid chromatography (DHPLC) is used for the separation of the heteroduplex and homoduplex strands. The mutation analysis with DHPLC can be almost totally automated with an autosampler on one end and a fraction collector on the other. Analysis is rapid (about 5 minutes per sample), and simple evaluation of data distinguishes between simple and multiple peaks in the elution profiles, allowing lengths as large as 1.5 kb of DNA to be analyzed. A disadvantage may be the recommended use of Pfu DNA polymerase, which, as a high-fidelity enzyme, allows sharper peaks but may be less successful in amplifying some regions.

Short Tandem Repeat Profiling

A short tandem repeat (STR) is a type of DNA polymorphism that occurs when a pattern of two or more nucleotides is repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 bp (e.g., $CATG_n$ in a genomic region) and is typically in the noncoding intronic, or upstream/downstream regions. By examining several STR loci and counting how many repeats of a specific STR sequence there are at a given locus, one can create a unique genetic profile of an individual. Currently more than 10,000 STR sequences in the human genome have been published. STR analysis has become the prevalent analysis procedure for determining genetic profiles in forensic cases. STR analysis in the field of forensics came into popularity in the mid to late 1990s. The STRs in use for forensic analysis are tetra- or penta-nucleotide repeats (4 or 5 repeat units) because these give a high degree of error-free data while being robust enough to survive degradation in nonideal conditions. Shorter repeat sequences tend to suffer from artifacts such as stutter and preferential amplification; several genetic diseases are associated with tri-nucleotide repeats, including Huntington's disease. Longer repeat sequences suffer more highly from environmental degradation and do not amplify by PCR as well as do shorter sequences.

The analysis is performed by extracting nuclear DNA from the cells of a forensic sample of interest and then PCR amplifying specific polymorphic regions of the extracted sample. Once these sequences have been amplified, they are resolved either by gel electrophoresis or capillary electrophoresis, which allow the analyst to enumerate the repeats of the STR sequence in question. If the DNA is resolved by gel electrophoresis, the DNA can be visualized either by silver staining or an intercalating dye such as ethidium bromide or, as in most modern forensics labs, by fluorescent dyes. Instruments built to resolve STR fragments by capillary electrophoresis also use fluorescent dyes. In the United States, 13 core STR loci have been selected as the basis by which an individual genetic profile can be generated. These profiles are stored in local, state, and national DNA databanks such as the Combined DNA Index System (CODIS).

Forensic reference materials are available. The DNA Profiling Standard is composed of well-characterized human DNA in two forms: genomic DNA and DNA to be extracted from cells spotted onto filter paper.

ASSAY VALIDATION CONSIDERATIONS

The difficulty in reproducing and validating existing and emerging SNP genotyping assays due to factors such as variation in performance of PCR thermal cyclers, efficiency of different enzymes, personnel, and the presence of PCR inhibitors in the sample matrix (discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩ for general NAT assays) can hamper appropriate implementation of the technologies. Also, in the clinical laboratory the use of in-house assay formats often makes comparisons between laboratories difficult. Incorrect diagnosis of a genetic mutation can have significant consequences, so accuracy of 99.99% or higher is essential for such assays. To determine the accuracy of a technology, the new procedure should be validated on multiple samples in which the genotype has been previously determined with a gold standard procedure, such as sequencing. Even with the most accurate procedure of analysis, sample preparation and amplification and detection procedures must be optimized to eliminate any potential inaccuracies.

Some genotyping errors can be minimized by careful planning of the laboratory procedures, the inclusion of well-defined controls, and increased automation. However, errors due to the processes used for genotyping are sometimes difficult to

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overcome and need to be taken into account. The types of errors and the frequency with which they occur differ between different approaches. Situations in which preferential amplification of one allele or nonspecific probe hybridization occur can all result in SNP miscalls. Additional unanticipated polymorphisms present within the primer/probe sequences can lead to amplification bias, highlighting the need for careful assay design and validation using alternative techniques. Limited and degraded samples can also result in preferential allelic amplification due to chance PCR priming events at low copy number.

It is preferable to have a no-call result, which would require the test to be repeated, than a miscall that provides incorrect results that are subsequently reported. Performance of replicate assays may also help to ensure accuracy. Data interpretation can also affect accuracy. Wild-type, heterozygous, and homozygous mutant results should be clearly distinguished from one another, and a well-defined measure of uncertainty should be attributed to them. Proficiency testing schemes and ring-trials go some way toward ensuring that individual assays are fit for the purpose for which they are intended for specific applications and that the staff performing them are competent. Sharing of technical information for assay design and sample preparation will also help. The availability of reference panels of well-characterized samples aids assay design and evaluation and allows sound interlaboratory comparisons to be made.

⟨1130⟩ NUCLEIC ACID-BASED TECHNIQUES—APPROACHES FOR DETECTING TRACE NUCLEIC ACIDS (RESIDUAL DNA TESTING)

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. This chapter covers the analytical procedures used to quantify residual DNA in biopharmaceuticals.

Process characterization and the theoretical safety concerns associated with process-related impurities highlight the need for residual DNA testing in biopharmaceutical products. The ability of a manufacturing process to remove residual DNA from a biopharmaceutical product is an indicator of the quality and consistency of the process and that the process is under control. Additionally, the cells used to produce a biopharmaceutical can be sources of a range of complex, heterogeneous, and potentially unsafe impurities, with host cell DNA among these impurities. For continuous cell lines, the potential risk of residual DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence of an infectious viral genome in the cellular DNA of the cell substrate. The oncogenicity activity of residual DNA could arise through its capacity to induce a normal cell to become transformed, which may lead to tumorigenicity. Although animal testing has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans. Residual DNA content, up to 10 ng of residual DNA per parenteral dose, may be considered for DNA originating from mammalian cell cultures, but the acceptable residual DNA content may vary depending on the source of the residual DNA and the route of administration of the product. One can address residual DNA in biopharmaceutical processes in two ways: 1) by validating clearance during process validation; and/or 2) by monitoring residual DNA levels through routine testing of the drug substance. Generally, 10 ng per dose is the accepted limit, by health authorities, of host residual DNA derived from mammalian cell cultures. The level of concern regarding residual DNA can be tied to its source and the route of administration, so the residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation with regulatory agencies. Regardless of whether routine testing of a drug substance is used to determine residual DNA content or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA are required. DNA amplification techniques, such as quantitative PCR (qPCR), are used most often for residual DNA testing because of their superior sensitivity and unique advantages (e.g., high specificity). The expectation is that the analytical procedure used to quantify residual DNA in biopharmaceuticals has a detection limit well below the DNA level allowed by regulators for biopharmaceuticals (often 10 ng/dose). Assays based on hybridization, DNA-binding protein, and qPCR are typically the techniques of choice because they can meet the sensitivity expectation.

SAMPLE PRETREATMENT

Analysis of residual DNA requires accurate quantification of picogram levels of DNA in mg (or larger) quantities of product, which may be in a variety of matrices. In certain circumstances, the sample can be analyzed neat in the analytical procedure with acceptable recovery and precision. When the product or other sample components interfere with the assay sample, dilution may be all that is required to overcome the interference, so long as the specified DNA content of the sample remains within the useful range of the analytical procedure. When sample dilution is not effective in reducing assay interference, it may be necessary to use more extensive sample pretreatment procedures, such as proteolytic digestion, chemical dissociation, or extractions. It may be necessary to use a combination of different pretreatment steps to remove interference to an acceptable level. Extensive sample manipulation can lead to losses of DNA or introduction of environmental DNA, and should be a consideration when using one or more sample pretreatment steps. Contamination with environmental DNA may only be a concern when using a residual DNA procedure that is not sequence specific.

Protein samples may only require digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to quantitatively recover the residual DNA. It may also be possible that the DNA is bound to the sample components, and chemical dissociation (e.g., detergents) may disrupt the binding, allowing sufficient recovery in the residual DNA assay. Residual DNA test procedures often use protein reagents and the use of a chemical dissociation reagent. These materials must be used at a sufficiently low level or removed so that the analysis is not compromised.

It may be necessary to extract the DNA from the sample to remove the inhibitory substances that are causing the reduced DNA recovery. Extraction procedures are typically based on precipitating the DNA from the sample or DNA-specific binding

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to a matrix (e.g., magnetic beads). Historically, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples before analysis, but the phenol/chloroform extraction technique might not be the best choice for the low levels of DNA typically found in biopharmaceutical samples. Because of these low levels, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if the phenol/chloroform extraction technique is used. Commercial kits are available and have been used successfully for pretreatment of residual DNA samples for improved recovery in the residual DNA assay. For example, some kits use a chaotrope (sodium iodide) and a detergent (sodium *N*-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol. Extraction of DNA from the sample, based on binding to a solid matrix, can be found in various formats. One of the most popular formats uses magnetic beads, where the beads are added to a sample with a binding solution to capture the sample DNA on the beads. The beads are then captured and held in the sample tube using a magnetic stand while the supernatant containing the interfering substances is removed and discarded. The beads are washed repeatedly using a magnetic stand and a wash solution. Finally, the DNA is eluted from the beads for the assay using an elution buffer, with the beads being removed from the sample preparation using the magnetic stand.

The sample manipulation involved with pretreatment may reduce the recovery of the residual DNA or introduce environmental DNA into the sample. Great care must be taken during any sample manipulations to avoid DNA losses or contamination. The addition of target DNA-spiked samples in the residual DNA assay is a common practice. The target DNA-spiked sample should not be confused with the internal positive control (IPC), which is typically a nontarget DNA added after the sample pretreatment step to detect the presence of PCR inhibitors and to evaluate DNA amplification during the analysis. The IPC may also be introduced before the extraction to improve the control of this step. A recovery of 50%–150% of the spiked target DNA is often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects or sample preparation method) make achieving a recovery acceptance criterion of 50%–150% impractical, then correcting the observed DNA concentration by using the load recovery percentage is also an acceptable approach.

HYBRIDIZATION-BASED RESIDUAL DNA ASSAY

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured, labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against autoradiography film for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. The level of hybridization can be measured using a phosphor-imaging system. If the probe has a fluorescent label, the intensity of the spots is determined using a fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semiquantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure.

1. The first step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease.
2. During the second step, the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane.
3. During the third step, the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample.
4. In the fourth step, the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

POLYMERASE CHAIN REACTION TECHNIQUES

Real-time qPCR is a procedure that is well adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe and primers used in the analysis are key to the

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procedure. The most common qPCR method for detection of this amplification is referred to as the 5′ nuclease assay. In this format, the probe has a reporter dye attached to one end and a quencher dye attached to the other end. A pair of DNA primers is also added to the reaction. During the amplification reaction, a thermostable DNA polymerase initiates DNA synthesis where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA, synthesizing new complementary DNA. While following the template DNA, DNA polymerase I cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe, DNA polymerase I will cleave the probe. The reporter dye is released into the solution and, in the absence of the quencher dye, the resulting fluorescence is measured. Repeating the reaction cycle results in an amplification of the fluorescence signal. The number of cycles required for the fluorescence measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing the fluorescence obtained from a sample to a standard curve, analysts can quantify the residual DNA in the sample.

Alternative Detection Strategies

A number of innovative detection strategies have been developed and commercialized beyond that described above. A few of the most common are as follows:

1. Intercalating cyanine dyes fluoresce after binding to double-stranded DNA. The amount of dye incorporated is proportional to the amount of target amplicon generated. These dyes are inexpensive and easy to use. The disadvantage of this technique is the lack of a specific probe to confer sequence specificity beyond that afforded by the primers, and the dye will also bind somewhat to single-stranded DNA and RNA molecules. Consequently, primer dimers or nonspecific products may affect the quantification. However, it is possible to check for the specificity of the system by running a melting curve at the end of the PCR run, based on the principle that every product has a different dissociation temperature and depending on the size and base content.
2. Other sensitive probes exist that contain a stem-loop structure with a fluorophore and a quencher at their 5′ and 3′ ends, respectively. The stem is usually six bases long, mainly consisting of cytosines and guanines, and holds the probe in the hairpin configuration. The "stem" sequence keeps the fluorophore and the quencher in close proximity, but only in the absence of a sequence complementary to the "loop" sequence. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target, leading to separation between the fluorophore and the quencher, and the probe fluoresces. The amount of signal is proportional to the amount of target amplicon sequence. The increase in fluorescence that occurs is reversible, because there is no cleavage of the probe. It is also possible to design the stem structure to add specificity to this type of probe. However, these probes are often expensive, and the signal can be weak due to the limited possible physical separation between the fluorophore and the quencher.
3. A variation on the second example described above uses a single-stranded nucleic acid sequence containing the specific PCR primers, the specific probe with a stem-loop tail separating a fluorophore and a quencher, and a blocking group. The stem-loop tail is separated from the PCR primer sequence by a "PCR blocker", a chemical modification that prevents the polymerase from copying the stem-loop sequence of the primer. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched, and an increase in signal is observed. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in close proximity. Enzymatic cleavage is not required, nor a separate probe hybridization step, thereby reducing the time needed for signaling. This can result in stronger signals and lower background with faster cycling; however, these probes can be quite expensive and complicated to design.
4. Fluorogenic minor groove binder probes are short linear probes that have a minor groove binder with a nonfluorescent quencher on the 5′ end and a fluorophore on the 3′ end. The minor groove binder prevents the exonuclease activity of the DNA polymerase from cleaving the probe. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close together. The probe is elongated when bound to its target and quenching is decreased, leading to an increase in fluorescent signal proportional to the amount of accumulated amplicon. These probes are also expensive and can produce a low signal-to-noise ratio.

Quantitative Multiplex PCR-Based Residual DNA Assay

An evolution of qPCR is quantitative multiplex PCR, where several pairs of primers and the corresponding probes are introduced in the reaction medium to simultaneously detect multiple targets. Benefits include higher throughput and better control of false-negative results, whereas disadvantages come from amplification and detection interferences, as outlined in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. One of the applications of this technique is a duplex qPCR, where the introduction of an exogenous DNA, called IPC (internal positive control, see *Sample Pretreatment* above), enhances confidence in the accuracy of the analysis when appropriately amplified. Multiplex qPCR is not used as often as single-target qPCR to assay host cell residual DNA in biopharmaceuticals.

RESIDUAL DNA TESTING POINTS TO CONSIDER

When developing a residual DNA assay, one should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. In addition, environmental, health, and safety aspects should be considered. Traditionally, hybridization assays were performed using phosphorus (^{32}P)-labeled DNA and autoradiography. Because ^{32}P decays quickly, probes prepared with ^{32}P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome. These issues with ^{32}P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this process represents a semiquantitative assay, but if the intensity of the spots is determined using a

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densitometer or other imaging system, the results may be quantitative. DNA-binding protein assays and qPCR give quantitative results. Quantitative assays are typically preferred instead of semiquantitative assays (e.g., older hybridization-based methods), because the results are considered more accurate and precise, which allows better process monitoring and control. Because of sample matrix interference, a sample pretreatment step is often required to obtain accurate and reproducible results. Pretreatment steps can influence the recovery of DNA, so it is usually necessary to design the assay with a spike-recovery control with an acceptance criterion to ensure assay performance.

In-house controls are usually prepared in the laboratory and qualified by ultraviolet spectroscopy, using standard techniques employed in molecular biology, to determine the DNA content and purity. The hybridization assay uses genomic and/or vector DNA, labeled randomly throughout the DNA, as the hybridization probe reagent. For this reason, the hybridization assay is specific for the source of DNA but is not specific for a given sequence. A synthesized probe, specific for a specific sequence, can be prepared and used in the hybridization assay if this level of specificity is desirable. The DNA-binding protein residual DNA assay is not sequence specific and hence not specific for the host DNA. Therefore, laboratory personnel should avoid contaminating samples for this assay with environmental DNA before denaturing the DNA; otherwise, the DNA result may be falsely elevated. The qPCR probe has the advantage of being sequence specific, but this creates some special challenges for development of a qPCR residual DNA assay. The qPCR-specific sequence must be a stable sequence within a suitable region of DNA. The recovery of the target sequence must consistently represent the recovery of all the residual DNA. Biopharmaceutical manufacturing processes may typically include operations that shear DNA into smaller fragments, and this must be taken into consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. When bridging from one DNA assay technique to another, a thorough understanding of the DNA analyte is critical. Some assays can detect both single-stranded and double-stranded DNA, whereas some can only detect double-stranded DNA (e.g., some fluorescent dye-binding assays). There are assays that are not sequence specific, and those assays that are sequence specific can be influenced by the copy number of the target sequence present in the DNA. There are assays that require two or more antibody molecules to bind to the DNA fragment (e.g., DNA-binding protein-based residual DNA assay), and if the DNA fragments are too small and present in sufficient quantity, they can saturate the reagents and inhibit the assay (hook effect).

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and provides valuable characterization of the manufacturing process.

⟨1132⟩ RESIDUAL HOST CELL PROTEIN MEASUREMENT IN BIOPHARMACEUTICALS

1. INTRODUCTION AND SCOPE

Many medicinal products are produced through recombinant technology via a host cell (e.g., bacteria, yeast, or mammalian, insect, or plant cell lines). During the manufacture of such products, some amount of non-product, host cell-derived material will inevitably be introduced into the process stream. This process results in a mixture of the desired product and host cell-derived impurities, including host cell proteins (HCPs), and other process-related impurities that will be targeted for clearance through bioprocessing.

Residual HCPs have the potential to affect product quality, safety, and efficacy; therefore, the quantity of HCPs should be low. The product purification processes must be optimized to consistently remove as many HCPs as feasible, with the goal of making the product as pure as possible.

The primary concern with HCPs in biopharmaceutical products is their potential to induce anti-HCP antibodies that could induce a clinical effect in patients. In addition, HCPs may possibly act as adjuvants, which can induce anti-drug antibodies that can affect the safety or efficacy of the drug. A more extensive discussion of immunogenicity and its effect on preclinical and clinical studies is described in *USP* general chapter *Immunogenicity Assays—Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* ⟨1106⟩. HCPs can also have a direct effect on the quality of the product itself. For example, proteolytic HCPs, even in minute quantities, can cleave the desired protein product over time, reducing or eliminating biological potency or altering stability.

This chapter focuses on HCP immunoassays for recombinant therapeutic products. It does not address products such as vaccines or gene-, cell-, or tissue-based therapies, although the general principles discussed may apply to the measurement of HCPs in these products. The design and validation of immunoassays for HCPs involve unique and significant challenges due to: 1) the wide variety of possible HCPs in medicinal products; 2) the general use of polyclonal antibody reagents to detect them; 3) the lack of exactly matched standards for quantitation; 4) in some cases, a considerable effect from sample dilution effects; and 5) inherent limitations to measure single HCP species.

The chapter includes assay development strategies throughout the product and process development lifecycle, and it describes approaches to demonstrate that the assay is fit for use (e.g., illustrates unit operation clearance of HCPs, lot release). Because of the complexity of HCP immunoassays, careful development and characterization of critical reagents are required, particularly for the immunogen used to elicit the anti-HCP antibodies, the antibody reagent(s), and the assay HCP standard. Because HCP testing is an essential part of process development and product quality control, HCP testing is also discussed in conjunction with regulatory requirements and other considerations for guidance on an overall control strategy for HCPs. A brief outline of the general chapter follows:

1. Introduction and Scope
  1.1 Considerations for Manufacturing, Characterization, and Consistency
2. Terminology
3. HCP Immunoassay Methods

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3.1 The Assay Development Cycle
3.2 Development and Characterization of HCP Reagents
3.3 Immunoassay Method Development and Qualifying as Fit for Use
4. HCP Immunoassay Method Validation
4.1 Accuracy
4.2 Sensitivity and Assay Range
4.3 Sample Linearity
4.4 Specificity
5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement
5.1 Considerations for Electrophoretic Methods
5.2 Considerations for Western Blot Methods
5.3 Considerations for Chromatographic and Proteomic Methods
5.4 Concluding Remarks on Supporting Technologies for HCPs
6. Use of HCP Immunoassays for Process Development, Characterization, and Validation
6.1 Assays for Individual HCPs
6.2. Control Strategy
7. Summary and Conclusions
8. Bibliography

1.1 Considerations for Manufacturing, Characterization, and Consistency

Different cell-based expression systems are used to manufacture medicinal products, such as bacteria (*Escherichia coli*, *Pseudomonas fluorescens*), yeast (*Saccharomyces cerevisae*, *Pichia pastoris*), mammalian cells (e.g., Chinese hamster ovary (CHO), mouse myeloma cell line NSO, and others), insect cells (baculovirus-infected *Spodoptera frugiperda* cells), and plant cells (tobacco, *Arabidopsis*, rice). The particular HCP profile is unique and specific to the particular host cells under specific culture conditions and manufacturing processes. HCPs can vary in pI (~3–11) and hydrophobicity, and HCPs display a wide range of molecular weights (from ~5 kDa to at least ~250 kDa), depending on the host cell and manufacturing process used. The number of HCPs in upstream samples can run anywhere from several hundred to more than one thousand proteins, depending on the host cell and culture conditions. Although many cellular hosts have been used in biopharmaceutical manufacturing historically, the most experience has been gained using *E. coli* and the mammalian cells CHO, NSO, SP2/0, and human embryonic kidney cell line HEK293. The guidance in this chapter draws most heavily from the experience with these expression systems; however, general principles apply broadly to any host cell system.

In mammalian cells, the recombinant protein is typically secreted from the cells into the cell culture fluid (CCF), along with many of the HCPs. However, it has been observed that intracellular protein trafficking may not proceed in a normal fashion in production cultures. For example, proteins usually associated with intracellular organelles, such as lysosomes, may be found in the CCF of largely viable cell cultures, because the clones have been selected for maximum protein export. In addition, as some of the cells die, their soluble, intracellular proteins are released into the CCF. Some harvest operations also lyse cells; therefore, the resulting harvested CCF typically contains both secreted and intracellular HCPs. While this mixture of proteins incubates in the fermenter, additional changes in the HCP population may occur, for example, as the result of enzymatic activity (e.g., proteinases or sialidases).

HCP assays provide important information about the composition of the material entering the downstream recovery process and how each purification step affects HCP clearance. In some cases, HCPs can even bind to, and co-purify with, certain products. Process characterization and validation studies are needed to show which process steps remove HCPs and also to demonstrate the robustness of these steps for consistently removing HCPs. As such, HCP assays are an essential part of purification process development and help ensure manufacturing consistency. Lastly, reproducible and reliable HCP assays may be required to measure residual HCPs remaining in the drug substance (DS) used to make drug product (DP) that is delivered to the patient. HCP levels should be measured in: 1) preclinical lots used in toxicology assessment, 2) all lots during clinical development, and 3) process validation samples from the final manufacturing process. After approval, HCP monitoring may be required as an element of the control system. Subsequent sections of this chapter discuss in more detail the use of HCP assays in process validation and in a good manufacturing practices (GMP) control system.

2. TERMINOLOGY

To help establish a common nomenclature in the literature and with regulatory agencies, *Table 1* lists common terms with their definitions (indicating how they are used in this chapter) in addition to synonyms that have been used historically. Note that the term "platform" indicates that the same set of standards and reagents is used within a company to test a variety of products made from the same type of expression system (e.g., CHO cells) grown under similar upstream conditions. In the case of platform HCP assays, the antibodies to HCP are obtained from animals immunized with HCP antigens generated from a common upstream process that is applicable to many products, even if the downstream purifications are different. This approach allows the knowledge from prior products to be leveraged. Justification that an assay is suitable for a new product, using the same expression system and common upstream conditions, is therefore often relatively straightforward.

The HCP immunogen used to generate platform anti-HCP antibodies and used often as the assay calibration standard is, by design, comprised of a broad set of HCPs. In contrast, the qualifier "process-specific" indicates that the immunogen/standard has been prepared from a set of HCPs unique to a given process (either a unique upstream cell culture process or a unique downstream purification process). Process-specific assays are, therefore, limited in their utility, and each must be fully qualified for each process. Process-specific immunogens and calibration standards are, by intent, more narrow and specific to a given process. "Commercially available" assays produced by vendors are often derived from a combination of strains and harvest/purification procedures, and these assays are intended to have a broad application; but these commercially available assays are

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not specifically designed for a given manufacturer's proprietary cell line, and users do not have control over reagent availability and lot-to-lot consistency.

Table 1. HCP-associated Terminology

Term	Definition	Historical Synonyms
Commercially available	Available to the public for commercial sale; typically a combination of upstream isolates and corresponding antibodies made by the vendor and sold as reagents or kits.	Generic
Platform	The same set of an HCP standard and antibodies is developed with a company's proprietary host cell strain and used broadly within a type (e.g., CHO) across several products when the upstream conditions are similar.	Custom, in-house, proprietary
Upstream process specific	An assay designed from material where the upstream culture process deviates significantly from the platform. This is generally before any purification and may be applied to more than one product if these parameters are similar.	Custom, in-house, product-specific, proprietary
Downstream process specific	An assay designed from materials where the downstream unit operations are used to enrich the HCP population. This may be applied to more than one product if these parameters are similar. This is rarely used today and is not recommended except for certain products with exceptional downstream processing.	Custom, in-house, product-specific, proprietary
Assay for an individual HCP	An assay using a standard composed of an identified, single, known HCP and its specific antibody/antibodies.	Single analyte assay or Custom, HCP-specific
Coverage	Describes the assessment of how completely a population of polyclonal antibodies recognize the population of HCPs. The coverage assessment may be made on the HCP population used as the HCP antigen or from the product production culture.	
Qualification	Demonstration of suitability of analytical methods (including reagents used in these methods) for their intended application to a given process and in-process samples.	
Protein A/G-affinity purification	Affinity purification of antibodies with immobilized Protein A or G[a]	Affinity chromatography
HCP-affinity purification	Affinity purification of antibodies using immobilized HCP (antigen)[a]	Affinity chromatography, immunoaffinity chromatography
Null cell	The cell strain used for production that does not contain the product-specific genetic elements; includes untransfected parental cells and cells transfected with the expression vector but without the product gene.	Parental, blank, or mock-transfected cell
ng/mg	The numerical quantity (ratio) of HCP per product, where ng represents HCP mass and mg represents the product mass. It is calculated by dividing the HCP concentration (ng/mL) by the product protein concentration (mg/mL).	ppm[b]

[a] In some cases, both purifications are performed, typically the protein A/G first, then the HCP affinity.

[b] Although ppm has been used historically, it is not advised because this term is used to reflect mass per unit volume for other types of tests. It is recognized that ng is used conventionally as a value derived from interpolation from an HCP standard curve (in units of ng/mL), where the signal is reflective of antibody binding and, unlike the therapeutic protein concentration measurement, does not strictly reflect the mass of HCP that may be present.

3. HCP IMMUNOASSAY METHODS

Immunoassay methods rely on antibodies that recognize, as broadly as possible, the population of HCPs entering the downstream purification process; therefore, the sandwich immunoassay, designed with polyclonal antibodies, is the workhorse of HCP monitoring and quantitation. This assay format offers a combination of high sensitivity, specificity, throughput, automation potential, rapid turnaround, quantitative results, and low cost per assay that is unmatched by any other currently available assay technology. Other immunoassay formats (e.g., competitive immunoassays) may or may not be suitable, because they lack either the specificity or the sensitivity afforded by the sandwich format. Although these methods result in a single HCP value for a given lot, the number can give greater weight to HCPs for which high-affinity antibodies are present in the reagent(s)—and no or low weight to HCPs which are either not recognized or recognized by low-affinity antibodies in the assay. For these reasons, orthogonal measures of product purity are often needed. More details on these methods can be found in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement.*

The basic principles and design of immunoassays are discussed in *USP* chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩. The format that is most commonly used for HCP testing is the sandwich immunoassay with detection systems such as colorimetric, electrochemiluminescent (ECL), chemiluminescent, radioactive, or others. Homogeneous immunoassays, including competitive assays, where all of the reagents are combined at once and the binding occurs in a single step without washing, may be problematic due to antigen excess leading to antibody insufficiency issues (discussed later in the chapter); therefore, these formats should be used with caution. The heterogeneous sandwich immunoassay format described in chapter ⟨1103⟩ is generally preferred, because the dynamic range and sensitivity may be reduced in the homogeneous format. The formats, with their advantages and disadvantages, are discussed further in chapter *Immunological Test Methods—General Considerations* ⟨1102⟩. Data analysis is typically performed with a nonlinear fit of the sigmoidal curve generated by a wide range of standard concentrations, although some analyses may focus on the low end of the curve for greater sensitivity.

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3.1 The Assay Development Cycle

Figure 1 illustrates common assay development plans, depending on the reagents available at various stages. Fewer bridging studies are required when: 1) platform reagents are available, and 2) upstream processes are historically consistent. *Figure 1A* illustrates a scenario where platform methods are not available, and a commercially available assay is used up to the stage of process validation with appropriate assay qualification. For phase III and beyond, either a platform or upstream, process-specific method is preferred. A bridging study should be performed to support assay replacement. If the commercial assay is intended for phase III and post-approval, care must be taken to fully demonstrate that the assay reagents are applicable to the process HCPs. An additional consideration is that the reagents are from an outside vendor over whom the biopharmaceutical manufacturer has less control.

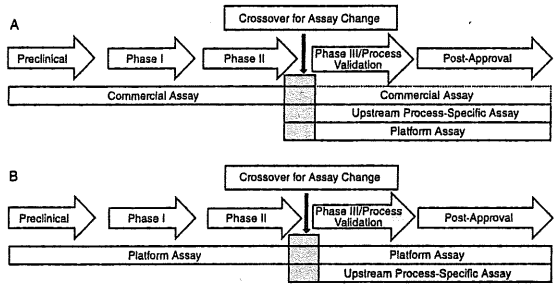

Figure 1A. If there is no platform assay before product development starts. Figure 1B. If there is a platform assay before product development starts.

Figure 1B suggests that a platform assay can be used through all stages of product development if already available when product development starts. The platform assay should be qualified for each new product. Switching to an upstream, process-specific assay may be necessary for phase III or process validation and beyond, if the cell culture process is significantly changed from the platform process, and may introduce significantly different HCP populations. A bridging study should be performed to support assay replacement.

The limitations of a downstream-specific HCP assay should be considered, because it may be tempting to think that HCP assays are improved by taking the HCPs from null expression through the first column(s) and immunizing animals with a downstream column pool. Historically, the logic was that the immunogen and standard would be enriched in those HCPs most likely to enter the recovery process and be in the final product. This strategy was based on the assumption that, rather than having thousands of irrelevant proteins in the immunogen, only those most likely to be in the process will be present in the immunogen; thus, the resulting assay will be focused on those HCPs of greatest interest. Potential concerns that may arise as a result of this approach are:

1. If only HCPs from a null cell run pool from the first column are used, then later changes during process development (e.g., changes in column run conditions) could invalidate the HCP assay. Thus, process development becomes very restricted and involves the risk of needing to develop multiple HCP assays for slight process changes (or the need to manage the uncertainty).
2. HCPs that co-purify with a product may do so because they bind directly or indirectly to the product protein. A null cell run of a column without the product protein would miss these HCPs.
3. Nonspecific adsorption to chromatography resins is not uncommon and, often, is not a reproducible phenomenon. Compared to the first passage, subsequent passage of the null cell run material over a column will likely produce a different set of HCPs after passage over a new column resin.

3.2 Development and Characterization of HCP Reagents

3.2.1 PREPARATION OF ANTIGEN/HCP STANDARD

As described in *1.1 Considerations for Manufacturing, Characterization, and Consistency*, the total HCP "antigen" is a complex population of proteins; therefore, when generating the HCP antigen/standard reagent, it is important to ensure that: 1) the calibration standard is representative of the cell line and manufacturing process, 2) its protein concentration is accurately quantified, and 3) the immunogen is administered in a way that generates polyclonal antibodies with reactivity to as many different HCPs as possible. The HCP antigen composition should also be comprehensive enough to tolerate normal process manufacturing changes during the life cycle of the product(s).

In addition to the uses above, the HCP antigen may also be needed to prepare the affinity column for purification of the antisera. If affinity purification is used, the quantity of HCP antigen needed should be carefully planned. The amount produced should be large enough to provide sufficient inventory for many years (often 10–20 years); ideally, for the whole life span of the product. However, the antigen preparation process should be performed and documented in a way that facilitates a potential resupply.

Because the HCP assay is used to test DS samples that contain trace HCP impurities, any cross-reactivity of the anti-HCP antibodies with the product may compromise the test method and yield biased results. Therefore, any contamination of the HCP antigen with product must be avoided to prevent the generation of anti-product antibodies.

3.2.1.1 Preparation of HCP antigen from mammalian cells: The majority of biopharmaceutical products produced today are expressed in mammalian cells, e.g., CHO cells. A typical process for the preparation of CHO HCP antigen is outlined in *Figure 2*.

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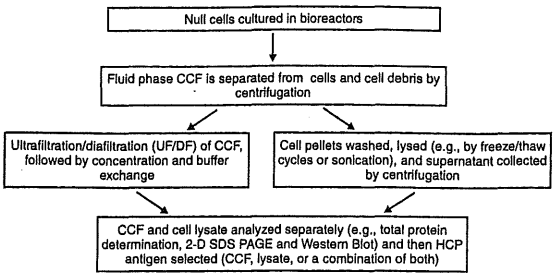

Figure 2. Example of mammalian HCP antigen preparation.

The first step is to establish a null cell that does not express the product gene, either by using non-transfected (i.e., parental) cells with a common origin to those used to make product alone, or to transfect them with the vector used to create the production cell line without the product coding gene (historically described as a "mock" transfection). The main advantage to the latter is that selective markers (e.g., dihydrofolate reductase or glutamine synthetase) are expressed. Alternatively, antibodies can be raised to selective markers independently. Another advantage is that mock-transfected null cells express selection markers and may be grown in cell culture conditions more closely resembling the manufacturing process. Using pools of mock transfected null lines which have not been cloned allows for the maximum potential genetic diversity and therefore gives the highest potential of generating a broad host cell protein population. However, these cultures, which are seeded with cell pools (not cloned), may demonstrate more variability from run to run. Both approaches [non-transfected (parental) and mock transfected] are commonly used. Both are also subject to the following issues: the cell culture process optimized for the production clone may not lead to similar viability and cell density of the null cells. In addition, in the absence of the stresses experienced by cells producing large quantities of product protein, the null cell lines may exhibit a different HCP profile. These changes in viability, cellular metabolism, and cell density may alter the HCP profile of the antigen, making it less representative of the manufacturing process. More about choosing appropriate HCP antigen preparation conditions is discussed below.

Once the null cells have been established, the HCP antigen is prepared in a bioreactor, cell culture flask, or cell culture bag reflective of the product or process cell culture conditions. A platform cell culture process may be applied, which represents a common manufacturing process for several products (e.g., CHO-derived monoclonal antibodies). Typically, the resulting HCP antigen is minimally processed to ensure a broad HCP spectrum, making the antigen suitable for HCP monitoring of multiple products generated from the platform process. A platform HCP antigen also may be produced by combining several null cell runs with slightly different cell culture conditions (e.g., allowing the culture to remain in the bioreactor for longer periods of time to vary the cell viability). This approach is used to obtain a broader HCP spectrum, and antisera raised against this immunogen may be suitable for HCP detection in products generated from a variety of slightly different processes. These reagents also may increase the robustness of the HCP immunoassay toward process changes.

Alternatively, the HCP antigen may be prepared using an upstream, process-specific approach in which the cell culture process is tailored to mimic the production process for a unique product or specific process. The advantage of this approach may be the high relevance of the resulting HCP antigen to a particular upstream process. As with platform reagents, because no additional processing purification steps are included, the antigen contains a broad range of HCPs, and the antigen will often remain suitable for HCP monitoring after downstream production-process changes. The disadvantage of this approach is that the use of the reagents typically will be limited to a single (or few) product(s) or process(es).

The HCP antigen can be produced from a representative small scale, pilot scale, or production scale run. There are pros and cons for each approach. Pilot or production scale may mimic the actual process best; however, normal variation between cell culture runs may not be reflected if only one run at large scale is applied. Conversely, several small-scale preparations can be pooled and may better reflect the run-to-run variability of the cell culture process. Regardless of the scale, the presence of product should be aggressively avoided, because its presence in the HCP immunogen will compromise the quality of the resulting immunoassay antisera.

After performing the null cell run, the HCP antigen can be prepared from the cell lysate, the concentrated CCF, or as a mixture of both as shown in *Figure 2*. If the antigen is prepared from CCF, the cells may be harvested at a time when production line harvest is performed or some days later, to allow for some of the cells to lyse and release HCPs into CCF and thereby broaden the HCP spectrum.

To prepare immunogen from cell lysate, the cells are harvested by centrifugation, and the cell pellets are washed and lysed (e.g., by using repeated cycles of freeze/thaw, or high pressure homogenization). Conditions are typically selected to mimic the production process (i.e., to prepare immunogen from CCF, cells and debris are removed by centrifugation, and the CCF is then treated by ultrafiltration/diafiltration). The buffer is exchanged (e.g., into PBS or HEPES), and the CCF is concentrated. The cut-off for filtration should be chosen to minimize loss of HCP, e.g., 10 kDa or less.

3.2.1.2 Preparation of HCP antigen from bacterial cells: Many of the above-described principles used in the preparation of mammalian HCP antigens also apply to prokaryotic cell culture. However, some unique challenges should be considered. In particular, manufacturing processes for bacterial expression system come in more varieties, such as those yielding concentrated protein deposits known as inclusion bodies, which can represent up to 95% of the total cell protein. HCP impurities are also present in the inclusion bodies, including bacterial membrane proteins, ribosomal subunit proteins, and cytoplasmic proteins, such as small heat shock proteins or chaperones. The formation of inclusion bodies in the production strain makes it more difficult to generate a representative HCP preparation from a null cell fermentation process. The null cell may not have the same HCP profile, and it will not generate inclusion bodies with which specific HCPs may co-purify, as described above. In other cases, HCPs have been created from periplasmic secretion systems, if the production process can be replicated in the null cell.

Because there is no obvious approach for overcoming these limitations, in practice the bacterial HCP antigen is usually generated from the lysates of washed cells, using null cell fermentations, that are grown and induced under conditions representing the upstream manufacturing process as closely as possible. As for any HCP antigen, it is necessary to demonstrate

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that the HCP profile is representative of the manufacturing process before use in assay development. This approach yields the full complement of HCPs expressed and hence is very broad.

Because most animals have been exposed to bacterial antigens and may have pre-existing antibodies to many bacterial cell proteins, it is important to characterize the pre-existing antibody responses, and if appropriate, consider the use of specific pathogen-free (SPF) animals. The presence of significant levels of pre-existing anti-HCP antibodies may confound the analysis of the antibody responses; therefore, it is important to screen preimmune sera from animals for pre-existing antibodies prior to immunization.

Null bacterial cells transfected with the expression vector contain chaperone genes that may be expressed at high levels (e.g., 5% of the total HCPs). Such overexpression of particular HCP impurities might necessitate the development of single-analyte assays for these particular impurities (see *6.1 Assays for Individual HCPs*).

3.2.1.3 Characterization of the HCP antigen: Several analyses are recommended before immunization: 1) protein content (total protein assay); 2) absence of product (as shown by Western blotting, immunoassay, and/or MS analysis); and 3) characterization by 1-D or 2-D-polyacrylamide gel electrophoresis (PAGE). Protein concentration is measured to establish the standard concentration for future use and to determine the overall amount of the prepared antigen. The most commonly used methods are the bicinchoninic assay (BCA), the Bradford assay, and amino acid analysis (AAA), although the colorimetric methods require a standard (e.g., BSA) that is not well matched to the (HCP) analyte. Absorbance at 280 nm (A280) may also be used although it is less specific for protein (e.g., nucleic acids will also be measured). These methodologies often provide similar results, but some methods may be more significantly affected by the presence of interfering substances than others. One should consider using two orthogonal methods to exclude a gross over- or underestimation of the protein concentration by one particular method. For more information, see general chapter *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩, which discusses the advantages and disadvantages of various protein analysis methods. The HCP concentration must be assigned with a scientifically sound approach that is used consistently for its lifetime. The lack of product can be assessed with gels, anti-product Western blots, or other suitable methods. Lastly, the 1-D and 2-D gels help characterize the pattern of HCPs, show that a broad spectrum of proteins is present, and show that a reasonable match to production is present. However, this can be challenging because of the presence of large amounts of the product, which can obscure HCP detection. Comparisons may be made between the specific populations of host proteins produced in the null cultures and production cultures using the orthogonal methods discussed in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement.*

3.2.1.4 HCP standard reference reagents: Frozen HCP material is usually stable for a very long time, but stability of the HCP standard reagents should be monitored over time because the assay lifetime can be very long. HCP standard stability can be confirmed by monitoring the HCP standard performance in the HCP immunoassay as well as in orthogonal methods.

Appropriate controls within the assay range may be established to monitor assay performance. Controls may be prepared from independent dilutions of the HCP standard, product samples or intermediate pools, or spiked product samples. However, there is a slight risk that control samples prepared as a dilution from the same HCP standard material can degrade at the same rate as undiluted HCP standard, and degradation of the standard will not be detected. To mitigate the risk, data from several standard curve parameters (e.g., signal, background, slope, coefficient of determination) are often assessed for each assay and used to support HCP standard stability. Using material different from the reference to prepare controls will help ensure that the degradation rate will be independent, and HCP standard degradation can be detected easily.

Assay control charts can be established and used to record reference curve parameters, control sample values, and lot-specific information for the critical reagents, such as labeled HCP antibodies. Acceptable ranges for the controls should be established on the basis of multiple runs (usually 20–30) and may be used as part of routine assay acceptance criteria. In addition, the values for % coefficient of variation (CV) of standard and control replicates are often a part of the assay acceptance criteria. Differences over time in HCP standard curve performance or HCP control levels, or an increase in assay variability, may indicate a lack of HCP standard stability.

3.2.2 PREPARATION AND CHARACTERIZATION OF ANTIBODIES

3.2.2.1 Preparation of antibodies to HCPs: The anti-HCP antibodies prepared are often used for many applications (Western blots, immunoassay) over a long period of time (e.g. the anticipated life of a product or platform). Therefore, material needs should not be underestimated. The recommended antisera quantity will depend on many factors including the intended use, the predicted quantity purified from a given volume of antisera, the amount coated on microtiter plates, and many other considerations. The choice of animal species used to generate anti-HCP antibodies is driven by the immunogen, the requirements for the particular method, and the preferences of the individual investigator. Rabbits, sheep, and goats are often chosen for immunization programs. In some cases, multiple animal species have been used. In such cases, using species more phylogenetically distant from mammals (e.g., chickens) have helped to generate anti-HCP antibodies to conserved mammalian HCP proteins. The choice of animal species may also be driven by a desire to either increase the amount of antisera available from one or several animals (goats or sheep), or to use a strategy to obtain a greater diversity of responses provided by the use of many more individual animals from a single species (e.g., rabbits). The number of animals chosen to generate the antiserum pool(s) depends on the species used and the duration of the immunization protocol. If smaller animals are used to generate the antiserum, more individual animals are generally needed (e.g., 10–20 rabbits). In contrast, if larger animals (goats or sheep) are used, investigators usually will immunize 3–10 animals per protocol. Typical immunization protocols provide approximately 100–150 mL of antiserum per rabbit or approximately 500 mL of antiserum per goat, and the total yield of Protein A or Protein G purified antibody is approximately 1 gram per rabbit or 5 grams per goat. Animals should be screened for pre-existing antibodies against drug substance prior to immunization. If positive results are found, then these animals should be excluded from immunization.

A portion of the prepared HCP antigen is mixed with an appropriate adjuvant (most commonly, Freund's adjuvant or in combination with incomplete adjuvant) and used for the immunization of animals. Each animal receives an initial priming immunization, followed by multiple (often 4 to 8) booster immunizations, to mature the immune response and generate high-titer, high-affinity antibody responses. Antiserum is collected from each animal before the initial priming immunization

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(time 0) and at appropriate intervals (usually 10–14 days) after booster doses. Four to six bleeds are usually collected from each animal. However, the duration of the immunization protocol may be extended by increasing the number of booster immunizations, making it possible to collect additional high-titer antisera. Other immunization strategies may also be helpful (e.g., cascade immunization or size fractionation of the HCP immunogen).

Investigators should perform titer or Western blot analyses of the individual test bleeds before antiserum pooling to screen for and remove sera that 1) have low titer, 2) display an immunodominant immune response to a small group of HCPs, or 3) have nonspecific binding characteristics or anti-product reactivity. This evaluation should also be performed on the final antibody pools to demonstrate broad coverage of HCPs and a lack of binding to the therapeutic product. In addition, some laboratories have found it useful to evaluate antibodies for aggregates using size exclusion chromatography as described below. Acid elution conditions may cause some antibodies to aggregate, and multimers of this type, particularly when labeled with either biotin or horseradish peroxidase, may lead to increased assay background. In addition, some laboratories include a final process-scale size-exclusion chromatography step to reduce aggregate levels to low levels (e.g., <5%).

Multiple approaches to the purification of antibodies from antiserum pools have successfully generated antibodies suitable for use in HCP immunoassays. Antibody purification is often performed by Protein A, Protein G, or HCP column affinity chromatography. The application of coated magnetic beads can also provide benefits in some scenarios. The choice of Protein A or Protein G is driven by which animal species was used to generate the antibody. Manufacturers provide standard protocols for these routine antibody purifications. When affinity purifying anti-HCP antibodies, materials such as chromatography resins, which may have been used previously for other products, should be avoided, and additional cleaning cycles should be in place to minimize carryover. Ideally, manufacturers should use a resin that has never been exposed to a DP. Before the affinity purification step, some laboratories also include an initial 50% ammonium sulfate precipitation of the crude antiserum to enrich and concentrate the antibody fraction and thereby extend the performance and duration of use of affinity columns. The use of Protein A or Protein G purification strategies generates reagents in which the anti-HCP-specific antibodies compose a smaller proportion of the total antibody. In contrast, HCP affinity purification strategies selectively purify anti-HCP-specific antibodies from the antisera or antibody-enriched preparations. In this case, the HCP antigens are coupled to an activated resin, and the antiserum is purified over the column following established protocols or the resin manufacturer's instructions. The purified antibodies may be further purified by size-exclusion chromatography to remove aggregates, then dialyzed, concentrated, divided into aliquots, and stored frozen (typically –70° or colder). Neat antisera should also be stored frozen (see ⟨1106⟩ for additional information regarding storage of serum samples).

Affinity purification of specific antibodies using HCP immobilized on a column requires careful management of the column preparation and use so that it can be reproduced reliably for future purifications. In addition, if reused, appropriate resin storage and regeneration procedures should be evaluated and controlled to avoid degradation during storage. Done properly, it is a reliable, reproducible process that has led to consistent anti-HCP antibodies and HCP immunoassays. After loading the column with antisera, an optimized wash procedure is critical. Because the preparation is a mixture of antibodies with differing affinities, extensive washing may remove low-affinity antibodies. Similarly, loading large amounts of anti-HCP antisera onto the column may result in high-affinity antibodies, displacing low-affinity antibodies if they recognize epitopes that are in close proximity. In either case, the low-affinity antibodies may be removed. In theory, higher affinity antibodies may also be more challenging to elute. Whatever load and wash conditions are initially selected for the antigen-specific affinity purification, these conditions need to be maintained in future production lots of the antibodies. A comparison between these two purification strategies is shown in *Table 2*. Both approaches are commonly used.

Table 2. Antibody Purification Strategies

Type of Purification	Pros	Cons
Protein A or G Affinity Column Chromatography	Robust and reproducible for quickly separating IgG fraction from other serum proteins	Lower proportion of antibodies specific to HCPs and may have a higher amount of low affinity antibodies
HCP Affinity Column Chromatography	Enriches for specific high affinity anti-HCP antibodies; excludes nonspecific antibodies and serum proteins	More skill and documentation required to make and maintain resins consistently. Some high affinity anti-HCP antibodies may not be eluted in usable form and may not be recovered

3.2.2.2 Characterization of antibodies to HCPs: Antibodies prepared for capture and detection are assessed independently and as part of the sandwich immunoassay pair. The concentration of the unmodified antibody is determined most commonly by absorbance at 280 nm, AAA, or BCA. Each approach is acceptable, provided it is applied consistently and (if colorimetric) is standardized similarly. It is important to determine the concentration of the capture and detection antibodies in each batch because they will be diluted to a certain concentration in the optimized immunoassay method. The detection and capture antibodies are characterized by their performance in the immunoassay method(s). The best label to antibody conjugation ratio should also be empirically evaluated primarily based on the best performance in the assay. This optimal ratio should be targeted in the future when new labeled antibodies are made. Finally, although antibodies are generally very stable when stored frozen (e.g., –70°), investigators should ensure storage integrity and determine a defined period of use. This can be accomplished by continuous monitoring with trending charts or by requalification experiments at specified time intervals.

The quality of the antibody pair (capture and detector) is often evaluated in two ways:

1. Show that the antibody pairs are specific and sensitive in an immunoassay format for the HCPs present in a series of samples taken from the unit operations of a given purification scheme, including the DS. This topic is also addressed in more detail in *4. HCP Immunoassay Method Validation*. In concept, the ability to detect and measure HCPs (immunoreactivity) in a series of actual process samples from a given process for a product in development is demonstrated with data showing: 1) sequential clearance as the product is purified, 2) sample linearity throughout a dilution series, and 3) lack of cross-reactivity to product or matrix. The reduction of HCPs during the purification process is determined by clearance factors where the HCP content (in ng/mg) at each step in the process is divided by the amount in the prior step. Typically, starting samples from the cell culture may have HCP levels ranging from several hundred

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thousand to several million ng/mg, and final products may have HCP levels ranging from <1 to 100 ng/mg (showing many logs of clearance). When these trends are observed, they suggest that the process and the assay are functioning effectively, and the antibodies used in the immunoassay have suitable quality attributes. If these trends are not observed, it may not necessarily reflect a problem with the antibodies, because it is also possible that the purification process is not effective.

2. Show that a broad range of HCPs in the calibration standard is recognized (i.e., that the coverage of the HCP population is adequate). Coverage is evaluated for at least the capture antibodies using 2-D gel Western blots or immunoaffinity fractionation of the total HCP population by immobilizing the anti-HCP antibodies on a column. In addition it is recommended to test the coverage of the detection antibody if different coating and detection antibodies are used. The benefits and limitations of using either 2-D gels or immunoaffinity fractionation to demonstrate coverage is discussed more fully below. Whichever method is used, the extent of coverage must be addressed in qualifying the immunochemical reagents.

HCP coverage evaluations help assess the ability of the antibodies to recognize a wide range of HCPs in the calibration standard and those present in in-process and DS samples. Two methods (2-D gels followed by Western blot analysis and immunoaffinity purification followed by 2-D gel analysis) are in fairly common usage, and both have the limitation that they tend to underestimate the true antigen binding in immunoassay methods but may have value in comparing two preparations head-to-head (or a platform to a commercial reagent). Both procedures use reducing 2-D gel electrophoresis to separate HCPs in the HCP standard or in early process sample(s) (e.g., harvest). Numerical coverage comparisons should be used with caution because of the many method variables and the art required to reproduce results, even with the same reagents in the same laboratory. Because of this variability, the results are best evaluated qualitatively. Comparisons of batches (or sources) of antibodies should be done side by side as much as possible to determine if antibody lots are comparable or if one is superior to another. One approach, the SDS-PAGE/Western, is essentially a comparison of the number of spots present in the immunologically stained membrane versus the number in a duplicate gel (or blot) stained for total protein (e.g., by fluorescent dye or silver; see also *USP* chapter *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩). Differential staining of each antibody preparation may power the analysis, because it can be used to analyze the same 2-D gel. The second approach, immunoaffinity binding/SDS-PAGE, involves comparing the flow through and eluate to the load from the HCP calibration standard (or early process sample) passed over a resin to which the capture antibodies have been covalently immobilized. The resin is washed after loading and before elution to remove nonspecifically bound HCPs. For the purpose of sample comparison, difference gel electrophoresis (DIGE) technology could be helpful. *Table 3* lists the advantages and disadvantages of these two coverage methods.

Table 3. Comparison of Coverage Methods

	Pros	Cons
2-D SDS-PAGE/Western blot	Good separation of individual HCPs allows for individual spot counting.	HCP antigens are denatured, may not represent what is seen in immunological assays (e.g., ELISA).
		High variability—numerical "percent coverage" values vary widely with the same material tested within a single lab and between different laboratories.
		Transfer efficiency of a broad range of HCPs difficult to optimize, leading to underestimates due to over-transfer through the membrane or failure to transfer from the gel, dependent on molecular weight.
		Matching spots between blots and gels are difficult and not standardized.
Immunoaffinity binding/ 1- or 2-D SDS-PAGE	HCPs bind to antibody resin in solution under native conditions similar to the sandwich immunoassay.	May underrepresent some HCPs if they are bound too tightly and do not elute, resulting in an underestimation of coverage.
	Analysis of spots in gels does not require immunoblotting and is simpler because the problems of transfer are avoided.	Preparation of the anti-HCP resin must be done carefully and may be difficult to reproduce. Results can be dependent on resin loading and elution conditions.
		Proteins at low concentration that only show up in Westerns will not be detected.

More information about the appropriate use of SDS-PAGE/Western blot methods and ways to minimize the disadvantages above can be found in the later section *5.2 Considerations for Western Blot Methods*.

An example of a suitable use of this approach is shown in *Figure 3*.

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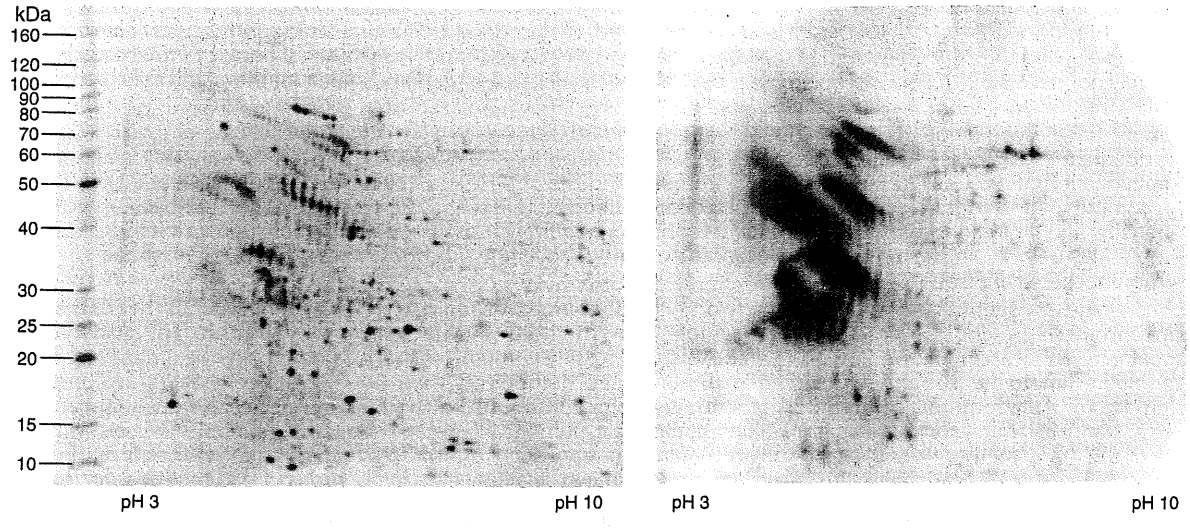

Figure 3. Left panel: 2-D IEF/SDS-PAGE analysis of representative CHO HCP calibration standard stained with a sensitive fluorescent stain. Right panel: Western blot analysis of the same gel shown in the left panel.

3.2.3 GENERATION AND QUALIFICATION OF REPLACEMENT REAGENTS

When the supply of HCP reagents are depleted or if their performance declines, new reagents may be needed. Often, new HCP antigens and new HCP antibodies are both generated at the same time. In theory, the new set of reagents should be created by following the same protocol that was used for the previous set of reagents to match the performance of the new and old assays as closely as possible and produce consistent data for a given program. In practice, however, it is not always possible (nor advisable) to generate the assay reagents exactly the same way that they were originally made. For example, if the manufacturing process has been optimized over time and the original platform assay reagents are no longer relevant, a careful assessment should be made. When such changes are required, the ideal way to confirm the quality of the replaced reagents is through characterization of antibodies (see below) and demonstrating that they can detect similar or greater HCP log reduction, from harvest to DS, using the same samples tested head to head with the original assay reagents.

When replacing an HCP antigen in which the current production process is essentially the same as the old process, the new HCP antigen should be prepared as similarly to the old as possible. It is important to compare the protein concentrations of the old and new HCP antigens side by side, using the same method to correctly assign the protein concentration for the new antigen and to ensure that the protein concentrations are comparable in the new and old HCP calibration standard. Characterization by 1-D and 2-D SDS-PAGE should also be performed as a side-by-side comparison of the old and new HCP antigens (see above) to be sure that gross changes in protein composition have not occurred.

For antibody replacement, the same animal species should be used, if possible. In cases where a better antibody response is desired, antibodies produced by different species may be evaluated. The selection of the species should take into account all the points discussed in this chapter. A side-by-side comparison of results from process samples using old and new antibodies in a sandwich immunoassay format should be included to demonstrate suitability of the replacement antibodies. Additional characterization by orthogonal methods (e.g., coverage using 1-D and 2-D SDS-PAGE) is recommended, as described above. Assay qualification or validation should also be considered after changing reagents.

To fully understand the effect of replacing HCP critical reagents on assay results requires testing of process samples in bridging studies using multiple samples from harvest, process intermediates from all appropriate purification steps, and the final DS. These studies can be very resource intensive, which underscores the importance of making sufficient quantities of HCP critical reagents at the outset (and minimizing replacement).

3.3 Immunoassay Method Development and Qualifying as Fit for Use

As noted in *1. Introduction and Scope*, HCP immunoassays serve two important functions in the development and control of biopharmaceutical manufacturing processes. HCP immunoassays serve as a measure of product purity, and therefore are potentially related to patient safety. They also serve as a measure of manufacturing consistency, and therefore they are reflective of process control. To meet these requirements, HCP assays are often validated and incorporated into the cGMP control system, which includes specific reject limits (in the DS as a Certificate of Analysis (C of A) method or as an in-process control). If the final DS is a conjugated protein, the testing should be done on the protein intermediate (before conjugation). In addition, HCP assays are used to guide process development and are often used to demonstrate robust process performance in the context of formalized process validation activities.

HCP immunoassays are often formatted as a sandwich ELISA, and multiple antibody labeling options are available (see ⟨1103⟩). Depending on the label chosen, appropriate conjugates and substrates are selected; examples are described in ⟨1103⟩. Most proteins can be biotinylated because of their lysine content and, because biotin is small, it is less likely to affect the protein's binding activity. Because antibodies are large and have many lysine residues, the conjugation ratio of biotin to antibody can be higher than that of smaller proteins. However, it is important to not over- or under-label the antibody (e.g.,

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with biotin or HRP), because this might lead to either less antigen binding or lower signal/sensitivity, respectively. Controlling and defining the labeling stoichiometry (e.g., mole of biotin to mole of IgG) is useful for making future batches consistently. Multiple ratios of any antibody label should be tested and optimized for performance in the immunoassay. Excess unconjugated reagents should be removed, either by dialysis, affinity purification, or by using a suitable desalting column.

Often, the standard is first screened to see whether a dose-response curve can be generated, and if so, in which concentration range of analyte. The results can also reveal a starting point for initial reagent concentrations that can be optimized further. If more than one antibody pair has been prepared, then each is tested to find the best performance (see *USP* chapter ⟨1103⟩ for more information). The signal (low calibration standard) to noise (background) ratios generated with each antibody candidate can be used to select the best pair. Typically, HCP immunoassays do not always possess a full dose-response curve with both asymptotes; therefore, for the purposes of residual HCP detection, the assay for DS release should focus on the low end of the curve.

3.3.1 QUALIFYING A NEW MANUFACTURING PROCESS WITH A PLATFORM HCP ASSAY

As noted earlier, if the upstream/isolation procedure is matched, then a new product produced in that system can benefit from a platform assay approach that can decrease qualification time and effort. However, in cases where there are variations that occur in optimizing the production cultures and the cell culture design space is not completely defined, it is important to understand whether these variations are significant enough to render the platform assay unsuitable. In these cases, the platform assay should be qualified experimentally to demonstrate that it is suitable for measuring HCPs from the new process. Assay accuracy, precision, sensitivity, linearity, range, and specificity must be evaluated (see *4. HCP Immunoassay Method Validation*). In addition, the range of HCPs in the platform HCP standard should be compared to those present in the new process. The antibodies should also be characterized using samples from the new process or product using approaches discussed previously.

The following approaches and concepts may be useful in determining if a platform assay is suitable for a product made with a new process:

1. Conduct a representative, small-scale null cell run grown under conditions used in the new culture process, and determine the protein concentration of the null cell run CCF. Assay the null cell run CCF at the same nominal concentration as the assay HCP standard using the HCP sandwich immunoassay, and compare the dilution curve from the null cell run CCF to the standard used in the assay. If the standard and new null cell run CCF curves are similar (e.g., in shape and amplitude, and the calculated HCP concentrations are within a factor of two), then the new process is considered not different from the platform, and a process-specific HCP assay is not usually needed.
2. With the CCF from the above null cell run from the new process, make a comparison to the HCP calibration standard using 2-D gel electrophoresis. This will compare the diversity of proteins produced by the two processes. However, be aware that new "spots" may appear in the 2-D gels that may not reflect immunochemical differences in the assay, for example, post-translational modifications or limited proteolysis. As such, the appearance of a few new spots or up-regulated or down-regulated spots in the process-specific null cell run is not a basis for invalidating the application of the platform assay. A qualitative assessment (e.g., versus a Western blot) is recommended.
3. Comparison of HCP levels at harvest in actual production runs (CCF samples) with the same product produced in the original versus the "new" process is also valuable. In general, immunoassay results within a factor of two are often considered similar, thereby confirming the platform HCP reagents.
4. HCPs in CCF from production cultures may also be estimated on the basis of total protein measurements. The total protein in the CCF is the sum of product (measured using a product-specific titer assay) and HCPs. Samples from harvests of the manufacturing run cell culture can be dialyzed, as long as they are sufficiently concentrated to make membrane losses negligible, to remove small peptides and amino acids, and then the total protein is determined (e.g., by amino acid analysis). Subtraction of the product concentration from the total protein provides an estimate of the non-product protein, HCP, in mg/mL. This value may be compared with the HCP concentration from the immunoassay. Comparable values (e.g., within 2×) indicate that the HCP platform is confirmed for the new process.

3.3.2 NEED FOR NEW REAGENTS AFTER PROCESS CHANGES

Major changes, such as substantial and unambiguous changes in cell culture conditions (e.g., moving from a serum-containing process to a serum-free production process), typically require production of new reagents and development and validation of a new HCP assay. In this example, the HCP reagents created using the old process recognize a number of serum proteins but may not recognize many new HCP proteins in the serum-free process. Because of both the process change and the assay change, different HCP levels will be observed when the same samples are measured for HCP in the old and new assays (i.e., the samples from the serum-containing process may have a high level of HCP measured by the old assay and low level of HCP measured by the new assay, whereas the samples from the serum-free process may have much lower HCP results by the old assay but more with the new assay).

If the assay was developed as a downstream, process-specific assay, changes in the purification steps can also require replacing the assay with a new one that is relevant to the new downstream process. This is the biggest problem with these very specialized assays and why platform-based assays are usually preferred, because they often do not require a new assay when the downstream steps are changed.

3.3.3. SAMPLE STABILITY

It is important to demonstrate that the sample handling procedures do not affect the assay results. This is particularly true when comparing results for various intermediate process pools. Occasionally, HCPs can precipitate during freezing or when the sample pH is adjusted from acidic to neutral. In addition, storage at 2°–8° or multiple freeze-thaws may result in loss of reactivity. The analyst may need to add specific stabilizing components (e.g., divalent cations) to the samples to ensure stability.

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4. HCP IMMUNOASSAY METHOD VALIDATION

HCP immunoassays for product purity assessment typically are part of the cGMP control system, either as an in-process test or on the C of A, and need to be validated appropriately. The validation approach depends in part on the types of samples that will be tested with the method. All validation parameters for a quantitative impurity test are needed when used for the final DS, whereas validation for in-process samples usually focuses on dilution linearity, interference, and precision. Regardless of whether the assay is an in-process or release assay (DS), action limits are valuable as a part of an overall control strategy. Readers are referred to ICH Q2(R1) guidelines and chapter *Validation of Compendial Procedures* ⟨1225⟩ for general expectations for assay validation, particularly those requirements for cGMP assay validation, and to chapter ⟨1103⟩ for sandwich immunoassays. Although the main focus of this section is on cGMP assay validation, many of the same principles apply to the assays used to demonstrate that process purification steps are suitable for their intended purpose.

This section will focus on those aspects of HCP immunoassays that are unique or require special attention and documentation. HCP immunoassays differ from more conventional immunoassays in that they are multi-analyte assays. There are potentially thousands of proteins in the immunogen and standard, and a correspondingly diverse set of antibodies is produced, yet only upstream CCF will contain a diversity of proteins that approximate the HCP calibration standard. Intermediate pools and final products typically contain progressively fewer of the HCP proteins that are present in the standard. As discussed previously, this is the aspect of accuracy that is compromised by HCP immunoassays, and why the mass unit ratios (ng of HCP per mg of DS) are not a literal "mass" relationship. However, below are common industry approaches that address these limitations as much as possible.

The validation summary below is for the final DS or a penultimate process step, whichever is chosen for the C of A method. It should be noted that for in-process testing, the focus is on method accuracy (spike recovery), dilution linearity, and precision. In the case of accuracy, some samples will have more potential interfering substances (other process impurities or additives) that may need to be confirmed as non-interfering (e.g., residual DNA, leached protein A, virus inactivators, chaotropes, and low pH buffers).

4.1 Accuracy

To assess HCP assay accuracy in the DS, at a minimum the analytical assay standard should be added to appropriate samples and accurate spike recovery demonstrated over the range of the assay. Matrix interference can come from buffer components and product, and the minimum dilution required for acceptable spike recovery (typically between 70% and 130%) needs to be determined for each sample type. Typically, spike recovery experiments are conducted with spikes using at least three and potentially as many as five different levels. Because samples are assayed at multiple dilutions, the investigator may spike and then dilute the sample, or dilute the sample and then spike the diluted samples. Spikes near the quantitation limit (QL) help to evaluate the accuracy and repeatability of the assay near the QL, which is where the measurement is often the most variable. A spike recovery of 50%–200% may be acceptable for a spike at or near the QL.

Although these are minimum requirements for assay validation, they should not be interpreted as demonstrating accuracy for any one specific HCP that may co-purify with the product. To accomplish that, comparison to a standard of that particular HCP species is needed; however, because this HCP is rarely known, this may not be possible. Tips for discerning these situations are provided below in the dilution linearity section and in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement.*

4.2 Sensitivity and Assay Range

HCP sandwich immunoassays often achieve standard curve sensitivity in the low single digits of ng/mL. For sample protein concentrations where the test dilution is 10 mg/mL, this implies that a sensitivity of about 1 ng of HCP per mg of product is possible. However, this sensitivity may be difficult to achieve for products with lower protein concentrations. It is important to highlight that the reported HCP ratio (ng of residual HCP relative to mg of product) is not really the ratio of masses implied by the unit ng/mg but rather "immunological equivalents" per mg of product.

The first aspect of determining QL is typically determined experimentally for each product in a given sample buffer matrix in spike recovery studies. The minimum recommended dilution (MRD) for the DS should be established by spiking the HCP standard in a sample dilution series. For example, an HCP spike of 10 ng/mL added to each of a series of solutions containing undiluted (e.g., 10 mg/mL) protein and serially diluted DS. Those dilutions in which the spike recovery is 70%–130% (50%–150% is also commonly used for levels near the QL) are considered acceptable. Typically, a proposed DS MRD is tested further to ensure that spike recovery is consistently achieved.

For the second part of the QL determination, the DS is tested at the MRD (if the DS samples have high levels of detectable HCP, a formulation buffer may be used). The QL is generally determined by the analysis of spiked concentrations of HCP and by establishing the minimum level at which the HCP can be determined with acceptable accuracy and precision. This study is typically performed at least three times, preferably by different analysts or on different days. For example, a spike of 3 ng/mL of HCP in a product protein concentration of 5 mg/mL has a QL of 0.6 ng/mg or 3 ng/mL if spike recovery is achieved in, e.g., at least four of six tests or if mean spike recovery criteria are met.

Typically, assays are set up to measure the range from a few ng/mL to >100,000 ng/mL. This range allows the analyst to test a variety of samples at multiple dilutions (see discussion of *4.3 Sample Linearity*) and allows for a practical assay that can accommodate in-process pools with highly differing HCP levels. For example, upstream samples may contain >100,000 ng/mL of HCPs and require large dilutions to obtain results in range of the standard curve.

For routine commercial DS manufacture where the protein product concentration is known and the process impurities are well understood, testing at a single dilution may be used for release. The results are reported as the ratio of measured HCP (ng/mL) to the product concentration (mg/mL) resulting in units of ng/mg. When the DS has undetectable levels the results are reported as "less than" the assay QL (ng/mL) divided by the product concentration (mg/mL; e.g., <0.6 ng/mg in the example

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above). Before setting this target concentration for testing, however, the dilution linearity of the samples should be well understood and a robust manufacturing process established. In the event that the level or species of HCPs vary run-to-run it may be necessary to test each sample at multiple dilutions (see below).

4.3 Sample Linearity

HCP assay nonlinearity is not uncommon for some samples and should be assessed for multiple batches of a given sample type (e.g., from several clinical DS lots or process validation batches) to ensure consistency and proper reporting of results. Because a single HCP (or a few individual HCPs) may co-purify with product, it is possible that particular HCP(s) will be present in excess of the available antibodies in the HCP immunoassay. This is possible in multi-analyte assays where only a limited surface area is available on assay microtiter plates or beads, and because thousands of different anti-HCPs are needed, antibodies to any one HCP will, of necessity, be limited. Furthermore, the relative amount of antibody to each separate HCP is also not controlled, so the binding capacity for each separate HCP differs. For samples exhibiting this behavior, the analyst must dilute the sample into the range of the assay, i.e., to a point where nonlinear behavior is no longer observed because the HCP has been diluted to a concentration where it no longer exceeds the available antibody. In some cases, the sensitivity of the assay becomes limiting, and one never reaches a dilution where the assay result is independent of the sample dilution. In such cases, the highest HCP ratio value (corrected for sample dilution) within the validated assay range should be reported. A detailed example of how to handle nonlinear dilutions arising as a result of antibody insufficiency follows.

Table 4 and *Figure 4* show data for three different samples tested in an ELISA that has a range of 1–100 ng/mL. In this example, all samples were diluted initially to 10 mg/mL (the MRD, where spike recovery had been previously confirmed), and then serially diluted using a twofold dilution series to below the assay QL of 1 ng/mL. Whereas *Sample 1* (diamonds) dilutes linearly over the full range tested, both *Sample 2* (squares) and *Sample 3* (triangles) HCP results plateau at higher concentrations of the sample. Saturation of the antibodies reflects antigen excess, and determination of the HCP value is made by diluting the sample into the range of linear dilution; visually estimated as <2 mg/mL for the squares and <1 mg/mL for the triangles. [NOTE—If large sample dilutions are required to get into the range of the HCP assay, consider making intermediate dilutions to limit dilution-related errors.]

Table 4. Raw Data for the Graph in *Figure 4*[a]

Product	Sample 1			Sample 2			Sample 3		
Conc. (mg/mL)	HCP conc. (ng/mL)	HCP ratio (ng/mg)	% max ratio value	HCP conc. (ng/mL)	HCP ratio (ng/mg)	% max ratio value	HCP conc. (ng/mL)	HCP ratio (ng/mg)	% max ratio value
10.00 (neat)	49 (neat)	4.90	83%	20 (neat)	2.00	<20	3.2 (neat)	0.32	22%
5.00	28.5	5.70	96%	16.5	3.30	54%	2.5	0.50	35%
2.50	12	4.80	81%	10	4.00	66%	1.5	0.60	42%
1.25	7.4	5.92	100%	7.4	5.92	97%	1.1	0.88	61%
0.625	3.1	4.96	84%	3.3	5.28	87%	0.9	1.44	100%
0.3125	1.6	5.12	86%	1.9	6.08	100%	<1	<6	NA
0.15625	<1	<6	NA	<1	<6	NA	<1	<6	NA
Reported HCP ratio value with guide and *n*	Guide 1 = 5.2 (*n* = 6); Guide 2 = 5.2 (*n* = 6)			Guide 1 = 5.8 (*n* = 3); Guide 2 = 5.3 (*n* = 4)			Guide 1 = 1.2 (*n* = 2); Guide 2 = 1.4 (*n* = 1)		

[a] Numerical data for three samples (also graphed in *Figure 4*) show differing degrees of saturation of the response at higher concentrations of product (and HCP impurity) tested. Two guides on how to interpret these data (guides 1 and 2) illustrate how the "linear range" of the assay is limited to the most dilute data points shown in *Table 4* and *Figure 4*. Note—Samples are always diluted until the HCP concentration is below the QL of the assay (in this example the QL is 1 ng/mL). Guide 1: All values within 20% of maximum value are averaged. Guide 2: Values are averaged as long as the CV of the values is <20%, removing less diluted samples first. A third guide might also be used that reports the highest value measured above the QL but the suitability of this approach should be very well demonstrated by method validation.

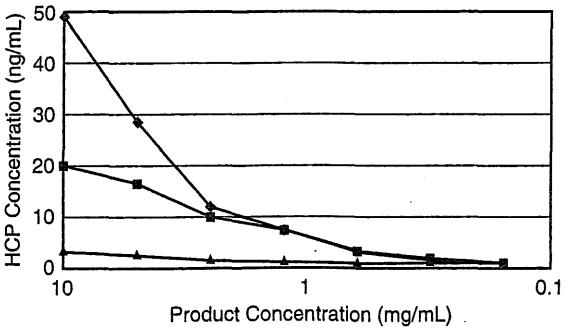

Figure 4. Twofold serial dilution of the three samples listed in *Table 4*. HCP concentration results are plotted against the product concentration in the samples, which serves as a measure of sample dilution.

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Because measurements near the QL may have higher variability and less reproducibility than measurements farther from the assay limit, usually (if possible) values within the sample linear HCP concentration response range are averaged. The procedure for which data points may be averaged must be specified in advance of running the assay and is typically documented as part of the test procedure. In the example shown in *Table 4*, two different guidelines are illustrated. *Guide 1* averages all values within 20%–25% of the maximum HCP ratio value. *Guide 2* averages values only if the CV for the resulting averaged values is <20%–25%. Although their data sets are not identical, both rules are justified, based on the argument that variations of 20%–25% are within the variation seen in assay replicates and reflect the underlying reproducibility of immunoassay methods. In this example, both methods give very similar results. In contrast, the third potential guide, which reports the highest value measured above the QL, would yield results that are at least 10% higher. In addition, the validity of the third guide would depend highly on the quality of the method development.

Homogeneous (one-step incubations without washes between reagent additions) sandwich immunoassays are more prone to antigen excess and resulting dilution nonlinearity problems and may exhibit "high-dose hook effects" in which samples with large amounts of antigen give inaccurately low readings, because the antigen excess prevents formation of the complete sandwich. Suitable use of this format should be confirmed experimentally for each sample type and could be acceptable if the samples are diluted to assay QL and lack of hook effects is confirmed.

Historically, some companies have used spike-recovery data produced in the validation of accuracy to demonstrate linearity. Although this demonstrates the linear dilution of the standard, it does not demonstrate the linear dilution of the HCPs in the samples. For the reasons of antigen excess discussed above, the linear dilution of the standard is a necessary condition but not sufficient to demonstrate linearity of the assay for different sample types.

Finally, although antibody insufficiency is generally believed to be the primary cause of a lack of dilution linearity, other potential causes are: 1) reactivity of the antibodies with product protein, 2) the presence of non-specific antibodies ("sticky" antibodies), 3) reactivity of the antibodies to non-HCP proteins present in samples (such as peptones or BSA), 4) HCP aggregation, 5) HCP standard dilution profile that is not representative of the in-process or final product HCP, and 6) sample matrix interference. Cross-reactivity and matrix interference are discussed below.

4.4 Specificity

Assessment of potential matrix interference in samples is addressed in section *4.1 Accuracy* and is primarily used to establish the lack of formulation interference (in the DS) and other impurities that are normally present (for upstream process samples). Another specificity issue to evaluate is the potential cross-reactivity of the anti-HCP antibodies with the product itself. Although this is possible, antibodies to HCPs that cross-react with product epitopes are rarely observed.

In those cases where cross-reactivity is observed, more false-positive assay signals are risks that must be managed. If cross-reactivity is suspected, it should be carefully and scientifically established. In the vast majority of cases, signals in HCP immunoassays are real, indicating the presence of HCP impurities. In particular, instances where the HCP(s) are noncovalently associated with product, and they co-purify, may give the appearance of cross-reactivity. However, because of the tremendous variety of antibodies in a polyclonal anti-HCP pool, when measuring residual HCP in the presence of excessive levels of product, some antibodies may still bind product, yielding a false-positive signal. In this case, it may be necessary to modify the assay or the anti-HCP reagent. If a platform HCP assay is used to test multiple batches (for the same program), and different levels of HCP between batches are observed, this is strong evidence that the HCP-positive signal is real and not due to cross-reactivity.

Evidence for cross-reactivity is often seen in a product that has been purified in a sequence of steps and where the HCP levels are not reduced by typical purification processes. The HCP/product ratio (ng/mg) should be used so that changes in the HCP/product ratio during purification can be compared. First, Western blots of DS are usually probed with anti-HCP antibodies to determine if HCP(s) are noncovalently associated with product. If bands are detected that are unrelated to the product, this suggests that cross-reactivity is not occurring, and further process development may be required if lower HCP levels are sought.

Alternatively, if the anti-HCP is recognizing a product-associated band, this suggests cross-reactivity. Caution should be exercised, however, because the product is denatured (by SDS-PAGE), and binding may be to epitopes not accessible when in the native, solution phase (i.e., in the immunoassay). In this case, a positive Western blot could be an artifact (conversely, a negative Western blot could also miss cross-reactivity, but this is less probable). Regardless, if cross-reactivity is suspected, it can be confirmed and corrected by either: 1) addition of a high-purity product (or related structures, such as human IgG from a different source, for a monoclonal antibody) to block the cross-reacting antibodies in the assay, or 2) depletion of the anti-HCP in the reagents using a product-affinity column made from highly purified product. In either case, it is important to ensure that the antigen used to block or deplete the serum is itself free of residual HCPs. This information is available from the Western blots probed with anti-HCP. If HCP bands are present, this indicates that a higher-purity product may be needed and can be prepared with orthogonal, lab-scale purifications. Sometimes reversed-phase HPLC can be effective in making this material, although this is not useful during product production because of its denaturing properties.

5. SUPPORTING TECHNOLOGIES FOR RESIDUAL HCP DETECTION, IDENTIFICATION, AND MEASUREMENT

Biopharmaceutical manufacturers need an integrated control system that offers assurance of overall product purity and manufacturing consistency. Typically, product purity is demonstrated by using a combination of methods, and this is very much the case for HCPs. In addition to the most common sandwich immunoassay format, other assay types provide valuable complementary information. *Tables 5*, *6*, and *7* provide overviews for commonly used methods for HCP detection, quantitation, and characterization. *Table 5* discusses electrophoretic methods; *Table 6* discusses Western blot methods; and *Table 7* discusses chromatographic and proteomic methods. Gel electrophoresis and immunoblots are also discussed in *3.2.2.2 Characterization of antibodies to HCPs*.

Orthogonal assay methods used to provide additional assurance of purity and the lack of HCPs may be used in any of three modes: "detection", "identification", or "quantitation". In the case of detection, orthogonal assays address the question, "Is

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there any protein other than a form of the product that the immunoassay may have missed?" If this is the case, as knowledge accumulates regarding the identity of the HCP(s), it becomes possible to perform a specific protein risk assessment and, if necessary, generate a standard and separate test for that particular HCP impurity. The orthogonal method may then be used in a "quantitative" mode by comparing the product sample with the appropriate HCP standard for the known HCP. For example, a quantitative HPLC-MS/MS method may be developed, based on monitoring peptides that ionize well from digests of the analytical standard protein and the sample.

5.1 Considerations for Electrophoretic Methods

Three electrophoretic methods that are useful in impurity analysis are 1-D and 2-D SDS-PAGE and SDS-capillary electrophoresis using non-gel sieving (CE-SDS). In all these methods, excess sample is loaded to ensure detection of trace protein impurities. The drawback is that excess product obscures impurities that migrate close to the product and cause interference. However, 1-D gels or CE-SDS may already be part of the GMP control system for monitoring product-related fragments; therefore, extending these methods for HCP monitoring does not involve additional testing. 2-D gels have been used to investigate the purity of the final product and to identify minor spots in the gels associated with either product variants or HCP impurities. The presence of a new band in the gel, or a new peak in the CE-SDS electropherogram, may represent either a new product-related substance/impurity or may reflect the presence of an HCP impurity ("process related"). In either case, an investigation is warranted. If not part of the routine control system, a 1-D gel is still an excellent method for extended characterization of important lots, such as registration lots, and for manufacturing investigations. Gels and CE-SDS are useful for confirming the absence of gross levels of HCPs (should they be present and missed in the immunoassay). Gels are typically stained with a high sensitivity stain, such as a fluorophore or silver. Ideally, the stain should be compatible with in-gel proteolytic digestion and subsequent analysis by mass spectrometry to identify the protein.

Proteins differ in their staining intensity with silver or fluorescent dyes; therefore, only semiquantitative estimates of the amount of protein in a band or spot are possible (e.g., glycosylated forms may not stain as intensely). Nonetheless, because these methods do not rely on anti-HCP antibodies, they provide an important orthogonal measure of purity.

Some proteins may appear in gels as multiple bands or spots that result from post-translational modifications or proteolytic clipping. To the extent that a single gene product is distributed to multiple locations in the gel, this further reduces the sensitivity of gels to detect impurities.

As discussed following *Table 7* on proteomic methods, it is often possible to excise bands or spots from the gels. These excised gel fragments may then be incubated with proteases to provide *in situ* digestion of the protein into peptides. These peptides may then be fractionated and sequenced using LC-MS/MS and compared with databases to identify the origin of the peptides as either product-related or HCPs.

Table 5. Analytical and Characterization Electrophoretic Methods for HCP Testing

Method	Use	Advantages	Disadvantages	Approximate Sensitivity for HCPs and Ability to Quantify
1-D gel, either reduced or nonreduced, stained with silver or high-sensitivity fluorescent dye.	Screening a large number of samples for consistency and presence of nonproduct protein bands. May be used as part of GMP control system.	High resolution separation; provides approximate molecular weight (MW) information; relatively simple to run. Side-by-side analyses of samples provide powerful comparisons for manufacturing consistency.	Large excess of product protein may obscure HCP bands. Different proteins show differences in staining intensities so only semi-quantitative. Limited capacity of gel lanes for total protein load.	100 ng/mg. Semiquantitative unless used with an analytical standard for a known HCP.
Large-format 2-D gel (e.g., 20 × 25 cm) stained with silver or a high-sensitivity fluorescent dye.	Screening a small number of samples for consistency and presence of unknown protein spots. Not suitable for routine use in lot release but may be used for characterization of specific lots or HCP standards.	High-resolution separation of trace HCP impurities from product. Provides approximate MW and pI information on protein spots.	Large excess of product protein may obscure HCP spots. Different proteins show differences in staining intensities so only semiquantitative. Highly technique-dependent results requiring skilled staff and sophisticated equipment.	100 ng/mg of total protein load). Semiquantitative unless used with an analytical standard for a known HCP.
CE-SDS with laser-induced fluorescence (LIF) detection. Fluorescently labeled samples are separated as either reduced or nonreduced samples.	Screening a large number of samples for consistency and presence of unknown protein peaks. May be used as part of GMP control system.	Rapid, high-resolution method to separate fluorescently labeled proteins.	Sensitivity may not be as high as a 1-D gel with a highly sensitive stain. Fluorescent labels are not specific for HCP proteins, so there is no distinction between HCP and product-related peptides. Difficult to extend to other measures, such as Western blots, or to identify protein peaks.	1000 ng/mg (0.1% of total protein load). Semiquantitative unless used with an analytical standard for a known HCP.

5.2 Considerations for Western Blot Methods

The Western blot technique is complementary to the sandwich immunoassay for demonstrating product purity and for characterizing the immunochemical reagents used in the immunoassay (see *3.2.2.2 Characterization of antibodies to HCPs*). When the anti-HCP antibody is used as a probe, it may be useful to: 1) detect HCPs that are noncovalently associated with the product and (potentially) missed in immunoassay, or 2) monitor an individual HCP. In other cases, a protein that reacts well in the immunoassay may not be detected in Western blot because a conformational epitope was lost following denaturation.

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Immunoblots require only one antibody to a given protein, whereas sandwich immunoassays require two. Previous work has shown that high-affinity antibodies that react with a given HCP can be more sensitive than silver or fluorescent dye staining of gels.

1-D Western blots of final product provide a simple reliable orthogonal method for demonstrating product purity. Although the same antibody preparation may be used in both the immunoassay and Western blot, for the reasons noted above, the blot may provide additional information on trace proteins (and miss others), hence its orthogonal nature. *Table 6* presents the advantages and disadvantages of 1-D and 2-D Western blot methods for these purposes.

Below is a list of the major considerations for optimization of SDS-PAGE/Western methods:

- 2-D-gel format and size: The larger formats (18-cm length or longer) resolve proteins better and come in a variety of isoelectric focusing (IEF) application modes and molecular weight dimensions (e.g., gradients). Total protein staining of various formats can be studied to optimize the separation in both dimensions and thereby find the balance that separates most (or the maximum number) of HCPs.
- The load on 2-D gels should be optimized to balance sensitivity needed (higher load) versus resolution desired (lower load). The protein load, ionic content of the sample, and speed of separation need to be balanced to avoid over-heating and to optimize resolution. The stains or substrates to be used will influence this choice. Typically, more is loaded for the immunoblot (e.g., 200 µg) than for the total protein-stained gel or blot (e.g., 50 µg for silver stains). The difference in load may confound the separation to some extent; therefore, finding the right balance will require experimentation.
- Signal development for both the total protein (gel or blot) and the Western blot requires significant experimentation (e.g., dye types, exposure times, buffer conditions) to optimize. For example, because it is important to detect as many bands as possible, sometimes the signal is amplified so much that the most abundant species obscure the others, or the background increases and contrast is lost. A related issue is standardization and calibration of the densitometer or imager, which must be fine-tuned to achieve consistency under experimentally determined conditions. Both reagent and instrument optimization can require significant time to reproducibly establish the optimized conditions where signal is maximized and background is minimized (see also ⟨1104⟩).
- The transfer and blocking reagents require experimentation to identify the best conditions (such as buffers, instrument power/time, temperature, and blockers).

Table 6. Analytical and Characterization Western Blot Methods for HCP Testing

Method	Use	Advantages	Disadvantages	Approximate Sensitivity and Ability to Quantify HCPs
1-D gel, Western blot	Screening a large number of samples for consistency and presence of unknown protein bands that react with anti-HCP antibodies. May be used as part of GMP control system.	High-resolution separation; provides approximate MW information; relatively simple to run. Side-by-side analyses of samples provide powerful comparisons of lots.	Requires high-quality anti-HCP antibody source. Large excess of product in the gel can lead to nonspecific staining of product bands. SDS-induced denaturation of proteins leads to loss of conformational epitopes.	Not quantitative for HCPs unless a specific protein standard is used. Sensitivity depends entirely on the quality of the antibody.
Large-format 2-D gel (e.g., 20 × 25 cm) Western Blot with chemiluminescence or similar high-sensitivity detection.	Screening a small number of samples for consistency and presence of unknown protein spots reacting with HCP antibodies. Not suitable for routine use but may be used for characterization of specific lots of product or characterization of antibody reagents.	High-resolution separation of trace HCP impurities from product. Provides approximate MW and pI information on protein spots.	Requires high-quality anti-HCP antibody source. Large excess of product protein may obscure HCP spots. SDS-denaturation of proteins leads to loss of conformational epitopes. Produces highly technique-dependent results requiring skilled staff and sophisticated equipment.	Not quantitative for HCPs unless a specific protein standard is used. Sensitivity depends entirely on the quality of the antibody, but 100 ng/mg is possible.

5.3 Considerations for Chromatographic and Proteomic Methods

Mass spectrometric techniques for the detection, identification, and quantitation of individual HCPs are rapidly evolving and leverage much of the technology developed for proteomics. Furthermore, as new genomic databases become available (e.g., the CHO genome), mass spectrometric approaches are more useful. These methods (see also *Applications of Mass Spectrometry* ⟨1736⟩ for additional information) often combine sample preparation such as reduction, alkylation, and proteolytic digestion, followed by separation (e.g., with reversed-phase chromatography RP-HPLC) before introduction into a mass spectrometer that fragments all proteins thus providing an amino acid sequence for each peptide. The resulting sequence information is compared to the product sequence to identify product-related fragments and to a database related to the host (e.g., *E. coli*, CHO) to identify HCPs.

A challenge for MS analysis stems from the overwhelming number of product-derived peptides relative to impurity peptides. One approach to address this issue of competitive ionization of the peptides (also called ion suppression of the HCP peptides by the product peptides), is to apply LC-MS/MS analysis on partially resolved HCP preparations (e.g., HPLC fractions). This purification reduces the product contribution to total ions in the mass spectrometer. Other approaches are in development. Recent technological improvements such as 1) chromatography resins able to resolve effectively using MS-friendly mobile phases, 2) improved interfaces to front end LC and/or IEF separation systems, and 3) mass spectrometers with higher mass resolution, accuracy, and faster scan rates, now make it possible to identify and quantify specific HCPs in DS with a high degree of confidence. Major challenges in terms of sensitivity and quantitation of sufficiently large sets of heterogeneous HCPs, cost, and QC-related issues remain to be met before this technology can replace immunoassays for the control of HCPs in DS.

As a characterization method orthogonal to the HCP immunoassay, LC-MS/MS data may be used in two ways. First, if the MS-based method does not find HCPs in samples that were also below QL in the immunoassay, then these orthogonal

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techniques can increase confidence that the HCP ELISA did not miss an HCP that has co-purified. The detection limit for many LC-MS/MS methods is currently in the range of about 10 to 100 ng of HCP per mg of product. It is therefore sufficiently sensitive to rule out a single HCP being present at a high level and is more sensitive than gels. Second, if an impurity is identified at a high level or one that is a safety concern, then one can assess whether the purification process needs to be improved to remove it, whether the current control system is adequate, and whether development of a specific assay is required. Absence of signal does not prove absence of HCPs, but once an HCP is identified, the sensitivity of LC-MS/MS can be enhanced. LC-MS/MS is complementary to other HCP technologies and is increasingly valuable as an orthogonal method. Detailed discussion of all the proteomic approaches to the analysis of HCPs is beyond the scope of this chapter, in part because the field is evolving so rapidly that any review would become out of date quickly.

Intact HCPs also may be separated using HPLC with UV detection. The profile has been used for comparing different lots of null cell runs, for example. Readers are referred to *Chromatography* ⟨621⟩ for the core science behind HPLC chromatographic methods. Because HCPs differ in abundance, and the chromatography may not provide unique resolution of all proteins, an individual HCP must constitute about 1% (or less with good resolution) of the total protein population for possible detection (even then, a given HCP may be obscured by product, depending on its retention time). Nonetheless, investigation of unknown peaks as a general practice and comparisons of the chromatographic profiles of null cell run HCPs may be useful in determining the similarity of HCPs produced under different culture conditions.

As with the electrophoretic methods discussed in this chapter, reversed-phase separation of proteolytically cleaved product samples with UV detection may already be included in a GMP control system as part of product testing. The presence of new or unexpected peaks in the UV chromatogram may indicate the presence of an HCP impurity, although this method is not overly sensitive (~1% impurity if not obscured by other peaks). However, because no new testing or sample preparation is required, this opportunity to use an orthogonal method for determining purity should be considered.

Table 7. Analytical and Characterization Chromatographic and Proteomic Methods for HCP Testing

Method	Use	Advantages	Disadvantages	Approximate Sensitivity and Ability to Quantify
Mass Spectrometry – HPLC/electrospray ionization (ESI)-MS/MS or cIEF/ESI-MS/MS or MALDI-TOF of protein digests	Identification of individual HCPs. May be applied to the characterization of a purified product lot, or null cell run HCP standards.	Allows identification and monitoring of individual HCPs. May be combined with gels to identify the individual proteins seen in gels.	Low-throughput method requiring significant sample preparation, skilled staff, and sophisticated instrumentation.	100 ng/mg and semi-quantitative if used in the discovery mode for unknown HCPs. 10 ng/mg (or better may be possible) and quantitative if used with an analytical standard for a known HCP.
RP-HPLC/UV or RP-UHPLC/UV. May be applied to intact proteins or proteolytic digests	Suitable for comparing null cell run standards if proteins are well separated. Larger proteins may need limited proteolysis or reduction to improve resolution.	High resolution, separates HCPs based on hydrophobicity and size; direct comparison of null strain and product strain is possible via overlays.	Difficult to find a stationary phase and mobile phase combination able to resolve and elute all HCPs. Co-elution may hinder quantitation of specific proteins. High concentration of product peak(s) may interfere with detection of trace HCPs.	10,000 ng/mg (1%). Semi-quantitative unless used with an analytical standard for a known HCP.

5.4 Concluding Remarks on Supporting Technologies for HCPs

A number of various hybrid analytical techniques have been attempted over the years. One example is fractionation of HCP standards by ion exchange, followed by RP chromatography, and then an immunoassay or MS analysis (2-D MS) on the proteins in the final fractions. Alternatively, "product subtraction" has been evaluated to remove the bulk of the total protein from final product samples before gel electrophoresis of LC-MS/MS analysis. These hybrid methods for coverage determination are very labor intensive, and they may introduce artifacts or biases of their own (e.g., antibodies to product do not typically remove all the myriad of heterogeneous forms). As such, they have not found widespread application, although in individual cases they may provide insight into the nature of product impurities.

Electrophoresis, Western blotting, and immunoassay remain the most widely used methods for HCP reagent characterization and for demonstration of HCP clearance by purification processes, because they are robust, sensitive, and can cover a broad range of protein impurities. Immunoassay and (increasingly) mass spectrometry are highly complementary and the most powerful methods for monitoring residual HCP levels in samples and confirming their absence in final DSs.

6. USE OF HCP IMMUNOASSAYS FOR PROCESS DEVELOPMENT, CHARACTERIZATION, AND VALIDATION

As described previously, although HCP testing has limitations as quantitative assays, if the immunoassay can rapidly and robustly detect most of the HCPs, then it is extremely valuable during process development, characterization, and validation. HCP clearance at each intermediate process step under a variety of conditions is valuable data. Application to process validation requires that the assay be demonstrated to be scientifically sound, suitable for its intended purpose, and appropriately documented. As stated earlier, process pools can differ widely in terms of buffer components, product concentration, and HCP composition. At a minimum, acceptable spike recovery of the HCP standard into in-process pool samples is necessary to qualify test dilutions of the various pools in the assay. In addition, testing each sample type at multiple dilutions is helpful during development to verify that: 1) results are within the range of the assay, and 2) nonlinearity in sample dilution is considered. In cases where the process is shown to clear HCPs robustly, process validation may also be used to justify not having a routine HCP test as part of the cGMP control system.

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Figure 5 illustrates data gathered during process development and validation and shows the dilution dependence for a number of in-process pools (*Pool 1* is after the first CCF purification step, *Pool 2* the next, and so on) through the process. *Figure 5* shows HCP ratio (in ng/mg) on the *y*-axis plotted against the product dilution in the test well on the *x*-axis. Each sample is serially diluted through three or four twofold dilutions. The starting dilution is determined by both the need to dilute samples with high levels of HCP into the range of the assay and the need to test only at dilutions where acceptable spike recovery has already been demonstrated. In *Figure 5*, the CCF has slightly more than 1 million ng of HCP per mg of product, meaning that a little less than one-half of the total protein in the CCF is product. The first column pool reduces the HCP level to just over 10,000 ng/mg. The second column has minimal HCP clearance; the third column reduces the HCP ratio to 1,000 ng/mg. The final column in the process reduces the HCP level by an additional 2 logs, resulting in a final HCP ratio of 10 ng/mg. All of the intermediate pools show HCP ratio values that are independent of the dilution of the sample.

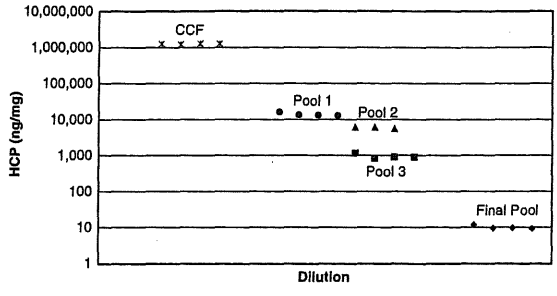

Figure 5. Example of HCP clearance by each purification step. Within a sample set, the first value is the most dilute in its dilution series.

Figure 6 presents a set of data for a different recovery process. As in *Figure 5*, the HCP ratio data for a serial twofold dilution series of each in-process pool sample are plotted. For CCF and the column 1 and column 2 pools, the HCP ratio (ng/mg) is independent of sample dilution (dilution linearity is achieved as in *Figure 5*). But after processing over the third column, distinct dilution dependence is apparent in the assay. This nonlinear dilution is likely associated with antigen excess relative to available antibody for the particular set of HCP(s) in the sample (i.e., a single or limited number of HCP species co-purify with the product). With product purification, more product must be put in the assay well for residual HCPs to be detectable in immunoassay. The product concentration in the assay may be increased with each purification step, and thus there is a similar increase in the concentration of the co-purifying HCP impurity. By column 3, the amount of the impurity likely exceeds the capacity of the available antibodies. This phenomenon is repeated in the final pool, which involves no HCP clearance but only buffer exchange by UF/DF. The reported value for these pools is the one obtained by diluting the sample to near the QL of the assay (the most dilute sample in the series) using a method described in *Table 4*, which is about 300 ng/mg in this case. Additional discussion of assay nonlinearity is found in the *4.3 Sample Linearity* subsection of *4. HCP Immunoassay Method Validation*.

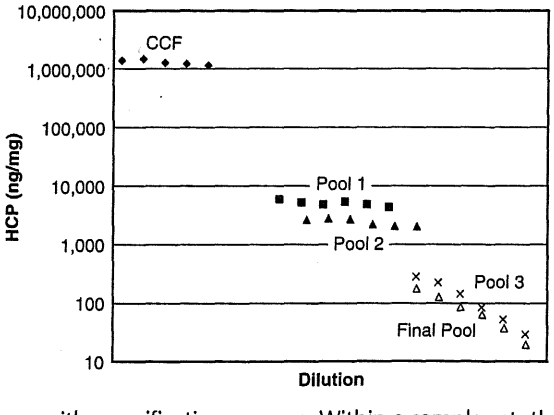

Figure 6. Example of HCP clearance with a purification process. Within a sample set, the first value is the most dilute in its dilution series.

6.1 Assays for Individual HCPs

In some cases, a manufacturing process might result in production of relatively high levels of a single HCP that can result in a bias in the reported results. As discussed above, in this situation the concentration of antibodies present in the total HCP assay may not be sufficient to capture all of that particular HCP present. In such cases, where a single, known HCP is present, another assay for that single HCP may be needed. For example, in the case of *E. coli* production cultures, co-expressing chaperones or other processing enzymes that aid in folding or disulfide formation can be produced in very high levels. In addition, these chaperones may be present in the immunogen used to develop the HCP assay reagents, in a conformation that is different from the way the protein is presented in samples. Thus, antibodies produced to chaperones not bound to product may not recognize, or may poorly recognize, product-bound HCPs. In this case, separate assays using appropriate antibodies

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may need to be developed. The existence of high levels of a single HCP is not unique to bacterial cell cultures and is possible with any cellular expression system. Another example is when a protein is intentionally added as part of the processing, then it is treated as a single HCP, and a specific assay will provide better data (than the total HCP immunoassay).

6.2 Control Strategy

6.2.1 TARGETS, ALERT LIMITS, AND REJECT LIMITS

In both United States and European Union regulations, and generally in other markets, HCPs are described as process-related impurities, and guidances state that their presence should be minimized by the use of appropriate, well-controlled manufacturing processes. U.S. 21 CFR 610.13 requires biological products to be "...free of extraneous material except that which is unavoidable...". ICH Q6B indicates that biologic product manufacturers should assess impurities that may be present and develop acceptance criteria for the impurities based on their preclinical and clinical experience. However, regulatory guidance also recognizes the inherent challenges involved in using assay-specific data and product- and process-specific reagents. Guidance states "The absolute purity of biotechnological and biological products is difficult to determine and the results are method-dependent" (ICH Q6B), and, "In the same manner, standardization of the analytical methods would be problematic since the reagents used are product and production system-related" [European Agency for the Evaluation of Medicinal Products (EMEA) 1997].

Although FDA guidance describes the need to test for HCPs, it also allows, through validation, removal of the release testing requirement, once clearance has been shown to be consistent. An expectation exists that, whenever possible, impurities that were introduced by manufacturing processes and remain in products should be below detectable levels based on a highly sensitive analytical method. However, an appropriately conducted clearance study performed as part of process validation can be an acceptable substitute for lot-to-lot (C of A) testing (FDA 1997), even when low levels of HCPs are present in the DS. In addition, "If impurities are qualitatively and quantitatively (i.e., relative amounts and/or concentrations) the same as in the DS, testing of the DP is not necessary" (ICH Q6B). This is the case for HCPs, the concentration of which in the DP may be inferred from the DS, because the manufacturing process does not introduce, or clear, HCPs when the DS is converted to the DP. Thus, in these instances, HCP testing of the DP is generally not required.

The overarching goal is to develop processes that minimize the quantities of residual HCPs in DSs. When using HCP immunoassays as part of a manufacturing control system, limits for acceptable results need to be established. The "target" for HCP in final product gives the purification development group an objective for the process, which may or may not be achievable. Antibody processes with highly selective affinity chromatography steps (e.g., using Protein A) are sometimes able to achieve a sensitivity of 1 ng of HCP per mg of protein. Target HCP levels determine the design criteria that are then used to develop the process.

Preclinical toxicology lots are the first lots for which official HCP values should be determined, because at this point in product development, the investigator is building the data set that will be used to support the safety of subsequent clinical studies. As the molecule advances through clinical development, the preceding studies are used to support the projected safety (and risk) associated with subsequent studies. Ideally, changes in HCP level and thus clinical exposure should decrease as the product moves through clinical development.

As part of an overall control strategy, the "reject limits" are those levels which, if exceeded, render the product no longer fit for use. During product development, knowledge about the process and the product increases; therefore, it is expected that reject limits can be tightened as one moves from toxicology material to phase I/II material, to phase III, and finally to commercial material. Each drug candidate profile and its clinical program are unique; hence, no single numerical limit is applicable to all.

Reject limits are primarily concerned with patient safety. Typically, one does not have specific data documenting "unsafe" levels of HCPs. Rather, acceptable levels for early stage products should be determined through a risk assessment (including non-clinical data, available literature, previous experience with products manufactured using the same or a similar cell line, etc.). For commercial products, the acceptable levels are also based on the experience gathered during clinical trials. Because the HCP impurity profiles may be influenced by the purification process, the registration batches used in the pivotal clinical trials must be representative of the to-be-marketed process. Reject limits for commercial product should be set to exclude HCP values that are outside the range of clinical experience upon which the risk/benefit decision was made for the biopharmaceutical.

Alert limits, trend limits, or action limits (different companies use different terms) reflect considerations of manufacturing consistency. In a commercial process, there should be sufficient manufacturing history to be able to define out-of-trend results. However, early in product development, where there is little or no manufacturing history, action limits may be used to set upper limits on the expected HCPs, based on experience with similar products for which there is a history, or in comparison to the target value. Exceeding these limits should trigger an investigation to determine whether the lot is releasable. Orthogonal measures of product purity are valuable in such investigations.

With the emergence of orthogonal measures of purity, any non-product related atypical signals should be evaluated. Efforts should be made to revise the purification process to remove any unwanted HCPs present at higher than desirable levels (based on, for example, clinical and non-clinical data, available literature, etc.), or a specific justification of its acceptability must be provided. This is accomplished as part of a risk analysis that considers the clinical exposure similar to that described above for immunoassays.

In cases where HCP assays are improved or changed due to unforeseen events, the reject limits may need to be modified, even increased in some cases (if the new assay has broader coverage). However, it is essential that samples be tested in bridging studies to ensure that no changes in quality would be missed in the new, improved assay. In some cases, improved assays give higher values, so bridging studies (head-to-head with original versus improved assay) will be needed to justify the new specification limit.

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6.2.2 SAFETY CONSIDERATIONS IN SETTING LIMITS

HCP specification limits in units of ng/mg are typically set using process capabilities and also on the basis of clinical experience (i.e., the levels used in trials where the clinical outcomes are known). Although there are several theoretical safety concerns about HCP impurities, the immunogenicity of the impurity is a primary consideration. An immune response may include formation of antibodies directed against the HCP or directed against the therapeutic protein, where the HCP is believed to have acted as an adjuvant. Readers are referred to ⟨1106⟩ for a more in-depth discussion of factors influencing immunogenicity. When patients receive more than one biotherapeutic protein with their associated impurities, immunogenicity is an even greater concern. Although high-dose products may contain a higher mass of residual HCPs per dose and this should be considered during risk assessments, the potential effect is not necessarily predictable of a clinical outcome. In some cases, very low levels of certain HCPs have been shown to have clinical effects, whereas high levels of other HCPs have had none.

Different limits may be set, depending on whether the product has only one or a very limited number of co-purifying HCPs or it has a multiplicity of HCPs, each present as a small fraction of the total HCP level in the product. This could lead to a separate assay and specification for individual HCP(s) that cannot be cleared from the process and are expected to be present in the DS and may have bioactivity.

The host cell origin can also be a consideration. It is possible that HCPs from human cell lines (e.g., HEK293 cells) may pose less immunogenicity risk than HCPs from more distantly related species because of sequence homology. However, if these proteins are normally intracellular, then they might still be immunogenic and seen as "foreign" by the patient's immune system. If such antibodies develop, then they could cross-react with endogenous human proteins and neutralize or render ineffective one or more necessary biological systems. As a consequence, arguments can be made on both sides; hence, the risks based on production cell line (microbial versus mammalian) are generally believed to be the same and cannot be practically informative *a priori*.

In addition to considering immunogenicity, the HCP may be bioactive and a risk. For example, a hamster homolog of a therapeutic protein expressed in CHO cells could be sufficiently similar to the product to co-purify. Alternatively, an anti-human cytokine antibody product expressed in CHO cells could bind to the homologous hamster cytokine, and the complex could co-purify with the antibody product. In both cases, the homologous hamster proteins could be biologically active when administered to patients.

7. SUMMARY AND CONCLUSIONS

Biopharmaceutical products should be as free of residual HCPs as reasonably possible. The industry standard for measuring HCPs is a sandwich immunoassay using polyclonal antibodies directed against the broadest possible spectrum of potential impurity proteins. Because the diversity and proportion of HCPs in the calibration standard is never matched exactly to the HCPs in any sample and due to the broad range of antibodies in the immunochemical reagents, different HCP assays will quantify individual HCPs to a differing extent. Thus, the single numerical value readout of the HCP immunoassay is "immunologically weighted". Highly immunogenic proteins will overquantify relative to the average population, and weakly immunogenic proteins will underquantify. Some proteins may not induce an immune response in the animal species used to generate antibodies, and the HCP immunoassay will be blind to those proteins. Because of this possibility, orthogonal methods for judging product purity also should be included in the control system. The best strategy is to use the HCP immunoassay in conjunction with other analytical tools.

Finally, one should use caution in extrapolating safe HCP values across products. No single specification limit will suffice. Differences in the biological properties of particular HCPs that co-purify with product, the route of administration, the patient population, and product/HCP dose all contribute to possible factors influencing patient risk. Over the history of recombinant biologic therapeutics, there have been examples where individual co-purifying proteins have been immunogenic, acted as adjuvants to increase the patient immune response to the therapeutic protein, or had biological activities themselves. Although these examples exist, they are rare, and overall there is an excellent safety record for biologics. In spite of the limitations and complexities of HCP assays, careful and diligent development and application of these assays have helped to ensure patient safety for millions of doses. HCP analysis continues to be a complex problem demanding time, resources, and careful thought by biopharmaceutical manufacturers with the goal of ensuring that products consistently meet the standard of having as low an HCP impurity level as can reasonably be achieved.

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Delete the following:

▲⟨1136⟩ PACKAGING AND REPACKAGING—SINGLE-UNIT CONTAINERS

SCOPE

This chapter provides guidance for the packaging and repackaging of single-unit containers, and for the use and application of unit-of-use packaging. Although the chapter is intended for use by drug manufacturers, repackagers, and pharmacists, the information in the chapter may also be useful for suppliers of packages and packaging components. For the definition of specific types of packaging, see *Packaging and Storage Requirements* ⟨659⟩.

SINGLE-UNIT CONTAINER

Single-unit containers that package a prescription drug to be dispensed directly to the patient are required to be child-resistant. Single-unit packaging intended for institutional or hospital use may or may not be required to be child-resistant. Single-unit containers that are child-resistant include supported blisters, such as separate, peel, push, and tear notch, and enclosed or in-card blisters, such as pull tabs and slide packs.

PACKAGING MATERIALS

Materials used to manufacture single-unit packaging containers include glass and plastic. Glass used as a primary packaging component should meet the requirements of *Containers—Glass* ⟨660⟩. Plastic materials as a primary packaging component should meet the requirements of *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. The test for moisture permeability may be carried out as described in general test chapter *Containers—Performance Testing* ⟨671⟩.

PACKAGING CLOSURE TYPES

Reclosables and nonreclosables may be used for solid, semisolid, and liquid dosage forms. Both must be packaged in compliance with the 16 CFR 1700.15 standards. The Poison Prevention Packaging Act (PPPA) of 1970 requires in certain cases the use of special packaging—child-resistant and senior-friendly. Child-resistant packaging protects children from serious injury or illness resulting from ingesting or handling hazardous products including drugs.

Because drugs packaged in unit-of-use packaging are intended to be dispensed to the consumer without repackaging by the pharmacist, the manufacturer or repackager is responsible for the special packaging of PPPA-regulated substances in unit-of-use containers (16 CFR 1701.1).

Reclosables

Reclosables are containers with suitable closures that may incorporate tamper evidence and child-resistance capabilities. Reclosables may be used for glass or plastic containers.

Nonreclosables

Nonreclosables are containers with closures that are nonreclosable, such as blisters, sachets, strips, and other single-unit containers. Nonreclosables may include packs such as cold-formed foil blisters, foil strip packs, and PVC/Aclar® combining multilayer materials that are thermo-formed or cold-formed foil blisters. Nonreclosables may be child resistant depending on the intended use and place of use. Household nonreclosables are subject to the PPPA as defined in 16 CFR 1700.14. However, because of some unit-dose designs, not all unit-dose packages comply with the PPPA.

UNIT-OF-USE

Unit-of-use packaging, when provided by the manufacturer, offers some of the following attractive advantages. (1) A dosage form can be dispensed to a patient in the manufacturer's original container, a practice that recognizes that the suitability of the container has been established on the basis of the manufacturer's stability studies. (2) The counting and repackaging of dosage units in the pharmacy is eliminated, thereby reducing the possibility of human error. (3) The pharmacist is able to affix the label for the patient onto the unit-of-use package and is free to use the manufacturer's expiration date as the beyond-use date. (4) The number of dosage units in a single unit-of-use package may be determined on a case-by-case basis. (5) Patient compliance is improved. (6) The unit-of use package can protect against counterfeiting because traceability of product is ensured through bar coding techniques and National Drug Code (NDC) numbers.

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Unit-of-use packaging, when provided by repackagers, offers the same attractive advantages as those offered by the manufacturer. However, unit-of-use repackagers should conform to all requirements as presented in *Good Repackaging Practices* ⟨1178⟩. There are a number of reasons why repackagers produce unit-of-use packaging, for example, (1) requests from institutions, (2) better inventory control, (3) reduced dispensing times, and (4) variations in some drug therapies.

The packaging of a unit-of-use system may be a multiple-unit or single-unit container. A unit-of-use system may contain a drug product in a liquid, semisolid, or solid dosage form (see also *FDA Guidance for Industry, Container Closure Systems for Packaging Human Drugs and Biologics*). [NOTE—The terms "unit-of-use package" and "unit-of-use container" may be used interchangeably.]

Unit-of-Use Labeling

The unit-of-use containers are labeled to include expiration dates, the manufacturer's lot number, the NDC designation, and bar codes as provided in *Labeling* ⟨7⟩ and *Good Repackaging Practices* ⟨1178⟩. Some of the advantages of having bar codes on the label include reduced medication errors, improved inventory control, and improved access to medication identity. The labeling covers information placed in the container by the manufacturer (see *General Notices and Requirements*). Acceptable labeling can range from full labeling, such as that for multiple-unit containers, to abbreviated labeling when the container is too small to include all of the text. Full labeling may also be provided on the carton if it is not present on the immediate container.

Unit-of-Use—Repackaging and Reprocessing

Unit-of-use containers are reprocessed or repackaged as instructed by the manufacturer. A unit-of-use package that is a blister package may not be reprocessed by a pharmacist once it has been deblistered from a unit-dose container (see *Labeling* ⟨7⟩, *Expiration Date and Beyond-Use Date*). Deblistering is the process of removing medication from a blister-type container. However, under current Good Manufacturing Practices (cGMPs) and tight quality controls, the manufacturer or contract repackager may repackage and reprocess unit-of-use containers.

Information from Manufacturers

The manufacturer should provide appropriate stability information that can be used to determine appropriate labeling, storage, and shipping statements that will properly inform patients and practitioners. The manufacturer may make other assurances based on product information on packaging and distribution arrangements. In the event that a product is not to be repackaged, the manufacturer may so state in the labeling. The manufacturer also includes labeling and information necessary for optimal handling by the practitioner and the patient. The labeling and information should be bar coded to eliminate medication error and promote medication traceability.

Responsibility of the Dispenser—Labeling

The labeling on a unit-of-use container also includes a label added at the dispensing stage by the pharmacist. Prior to dispensing the unit-of-use package, the dispenser shall add label(s) that provide the following information:

1. The name of the patient;
2. The name and strength of the drug product, the directions for use as prescribed by a doctor or health-care provider, and the name of the prescriber; and
3. Any storage instruction, beyond-use date, and other information as deemed appropriate by federal and state laws.

In the pharmacy setting, pharmacists are encouraged to use bar codes, in conjunction with computerized prescription orders, to confirm that the right drug is being dispensed to the right patient. Bar coding would minimize errors and create an opportunity for medication traceability and accountability.

Quality Control of Packaging System

The packaging system shall meet the general considerations for system suitability, protection, safety, and performance characteristics as described in *FDA Guidance for Industry, Container Closure Systems for Packaging Human Drugs and Biologics*, and in *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and *Containers—Performance Testing* ⟨671⟩.

REPACKAGING A SINGLE SOLID ORAL DRUG PRODUCT INTO A UNIT-DOSE CONTAINER

Repackaging of solid oral drug products, such as tablets and capsules, into unit-dose configurations is common practice both for the pharmacy that is dispensing drugs pursuant to a prescription and for the pharmaceutical repackaging firm. The following section contains minimum standards to be used as a guideline for repackaging practices.

Repackaging preparations into unit-dose configurations is an important aspect of pharmaceutical care and of optimization of patient compliance. For purposes of this chapter, there are two types of repackaging: the first involves pharmacies that dispense prescription drugs, and the second concerns commercial pharmaceutical repackaging firms.

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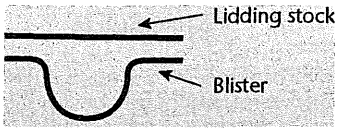
Materials

Blister packages offer a wide array of designs both in functionality and in appearance. Various packaging materials are used to create blisters that are tailored to provide optimum performance. The blister container consists of two components: the blister, which is the formed cavity that holds the product, and the lid stock, which is the material that seals to the blister, as shown below.



Schematic Presentation of a Typical Blister Pack

Because of the variety of blister films available, film selection should be based upon the degree of protection required. The choice of lid stock depends on how the blister is to be used, but generally the lid stock is made of aluminum foil. The material used to form the cavity is typically a plastic, which can be designed to protect the dosage form from moisture. There are widely varying degrees of moisture protection now available. For purposes of this general chapter, they are referred to as nominal, medium, high, and extreme moisture barrier properties.

POLYVINYL CHLORIDE

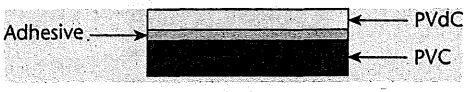
The most commonly used blister material is polyvinyl chloride (PVC). This material, which provides a nominal or zero barrier to moisture, is used when the product does not require effective moisture protection. PVC is available in a range of gauges and can be made opaque or can be tinted with pigments to block out specific light wavelengths.

The thickness of the PVC used is determined by the depth and size of the cavity to be formed. Because the plastic thins during the blister-forming process, care should be taken to ensure that the finished blister provides sufficient protection from light (if required) and that it is strong enough to adequately protect the dosage form. Common gauges of PVC used in the pharmaceutical industry range from 7.5 to 15 mil (0.0075 to 0.015 inch).

BARRIER FILMS

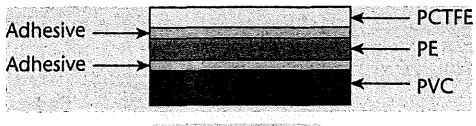
Many drug preparations are extremely sensitive to moisture and therefore require high barrier films. Several materials may be used to provide moisture protection. Barrier films commonly used in the pharmaceutical industry are described below.

PVC/PCTFE laminations: Polychlorotrifluoroethylene (PCTFE) film[1] is a thermoplastic film made from polychlorotrifluoroethylene fluoropolymer. The PCTFE film is laminated to the PVC by an adhesive layer between the PVC and the PCTFE film (duplex structure),



Duplex Structure

or by a layer of polyethylene (PE) between the PVC–adhesive and the PCTFE–adhesive layers (triplex structure).



Triplex Structure

By using various gauges of PCTFE film, medium to extreme moisture barriers can be obtained.

PVC/PVdC laminations: PVC/PVdC is a film in which the PVC is coated with an emulsion of polyvinylidene chloride (PVdC), as shown in the duplex structure pictured below.



Duplex Structure

The PVdC layer is specified in g/m² and can be constructed to provide medium to high barrier protection. The coating weights commonly used in the pharmaceutical industry are 40, 60, and 90 g/m², and the film is offered with or without a middle layer

[1] PCTFE film is available from Allied Signal (as Aclar®) and from other sources.

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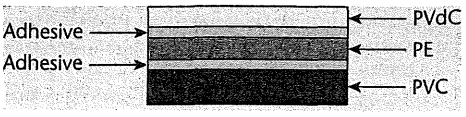
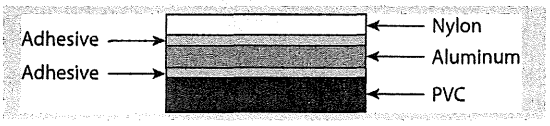
of polyethylene (PE), as shown in the triplex structure below. The polyethylene is used with heavier coating weights, such as 60 and 90 g/m², to improve the thermoforming characteristics of the blister cavity.



Triplex Structure

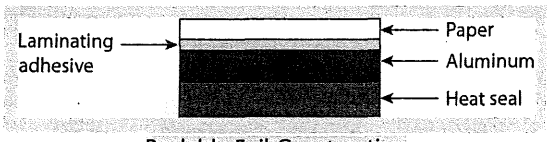
Polypropylene: Because of its morphology, polypropylene (PP) serves as a good moisture barrier, its spherulitic structure creating an arduous path for water molecules to traverse. Although not commonly used as a pharmaceutical blister film in the United States, PP provides an economical alternative to medium barrier materials and is used in Europe as an alternative to PVC.
Cold form foil: This material is used for products that are extremely hygroscopic or light sensitive. It is an extreme moisture barrier and consists of three layers: PVC, aluminum foil, and nylon.



Cold Form Foil

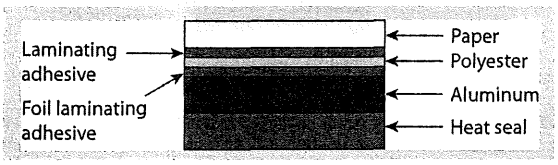
LID STOCK

The lid stock is sealed to the molded blister as described above. Different designs of lid stocks are available, and selection of a particular design depends on how the package will be used. Standard designs—peelable, child-resistant peelable, and push-through—are described below. The primary component of lid stock is typically aluminum, and its gauge varies from 18–25 µm (0.0078–0.001 inch). The side of the aluminum foil laminate in contact with the product provides the heat-sealable layer that forms the seal to the blister material. The heat-seal coating should be capable of forming an adequate seal with the blister film to which it is intended to seal. The materials used in the makeup of the heat-seal layer meet the requirements of 21 CFR 175 and 177.
Peelable: Peelable foil, commonly used in an institutional setting, consists of several layers, as shown below, and can be peeled away from the blister. [NOTE—For child-resistant peelable foil, a layer of polyester with the appropriate adhesives would be added.] With the peelable-foil lid stock, which is used in conjunction with blister tooling, a three-step process is required to open the blister.



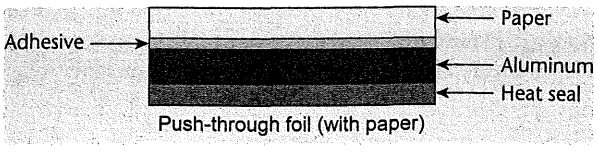
Peelable Foil Construction

First, the blister cavity must be separated from the rest of the blister card. Next, the paper and polyester layers are pulled back from an unsealed area. Finally, the product is pushed through the remaining aluminum foil. It is important to note that use of this type of foil structure helps make the package more child resistant. However, if child-resistant packaging is required, the package design should be tested in accordance with the protocol described in 16 CFR 1700, the Poison Prevention Packaging Act.



Child-Resistant Foil

Push-through: There are two commonly used types of push-through foil: one with a paper outer layer separated from the aluminum by a layer of adhesive, and one without paper. The paper outer layer serves as an aesthetic and makes it possible to print on the back of the blister.



Push-through foil (with paper)

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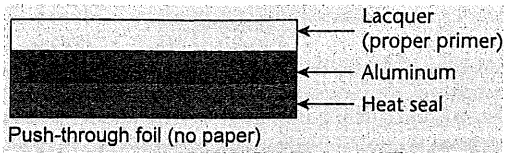
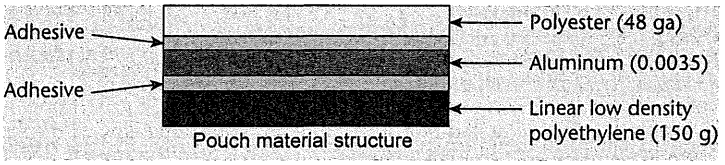


OTHER PACKAGE STYLES

Other types of packages used for unit-dose packaging of solid dosage forms are strip packs, pouches, and sachets.



PROCESS

Unit-dose packages can be formed and sealed in a variety of ways. Larger scale repackagers may use thermoformers that accomplish these functions in-line, while smaller repackagers may purchase preformed blister material. This section begins with an overview of the process involved in thermoforming a blister, the fundamental process that also applies to other unit-dose package types such as pouches. The overview is not intended to be all-encompassing, but it highlights the major operations along with their critical parameters.

Thermoforming a Blister Unit-Dose Package

The complete thermoforming process consists of four basic stations where the following operations occur: forming, filling, sealing, and finishing. Thermoforming requires the use of heat and air in forming the blister. The lid stock material is sealed to the blister cavity material for a defined time (the stroke of the machine) at the point where the heat plate closes on the two materials.

FORMING STATION

Prior to entering the forming station, the blister material passes through a heating unit where the blister material is heated uniformly in stages to ensure proper formation. Because different plastics have different softening points, careful attention must be paid to determining the proper temperature of the heating station, which often has multiple temperature zones. The temperature, based on the blister material used and on the speed at which that material travels through the heating station, is a critical parameter for optimal performance. At the forming station, the blister material is heated to the point where the plastic softens sufficiently to allow the cavity to be formed. The blister material is drawn from a reel-mounted roll (referred to as the web) and pulled through the machine. A splicing table is located at the reel unwind to provide room for a second roll of blister material to be readily available for splicing and resumption of the packaging process. An unwind device may be installed to aid in moving the blister material from the roll as adjusted for a specific index.

Once the blister material is properly heated, compressed air is generally used to form the blister cavity. Upper and lower forming dies close on the blister material as air is introduced, forming a blister that corresponds to the size of the cavity. A plug assist may be necessary, depending on the material and size of the cavity. The plug assist ensures a uniform thinning of the blister material to optimize the protective characteristics of the formed material. Once the blister material is formed into the desired blister configuration, it is advanced to the filling station.

FILLING STATION

The product is loaded into the blister cavity at this station. An automated filling device may be used, or the cavities may be hand filled. The critical parameter at this station is proper filling of the formed blisters.

SEALING STATION

At this station, the lid stock is sealed to the filled blister cavity, using heat and pressure for a defined dwell time. The critical parameters to be considered at this station are temperature, pressure, and dwell time.

The lid stock material is staged on a roll above the blister cavity and may be preprinted or printed online. Lot numbers and expiration dates may be applied at this point. Preprinted lid stock materials will require a print registration system to control the position of the printing relative to the blister cavity. The critical parameters at this part of the station include legible and correct labeling.

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FINISHING STATION

The finishing station encompasses all other steps in the packaging process, including embossing, perforation, and cutting. Embossing involves application of a lot number and expiration date to the package. Steel type is used to emboss information on the edges of the blister package. One of the critical parameters at this station is package integrity. It is important that the embossing, perforation, and cutting processes do not compromise the blister, lid, or seal. The quality of the embossing is another critical parameter in the process. The embossing must be legible and correct, and must include all required information.

Pouch Unit-Dose Packages

The pouch process is also a form, fill, and seal operation, but it does not provide a defined, formed cavity as does the thermoforming process. Although the equipment used to form pouch unit-dose packages may function differently from that described for thermoforming a blister, the main operations (form, fill, and seal) and critical parameters at those stations are quite similar. [NOTE—See the critical parameters defined in the section on thermoforming.]

The strip-pack process involves the drug product being dosed into a three-sided, formed pouch. Once filled with the drug, the machine seals the pouch, forming a strip of sealed unit-dose pouches. The basic flow of the process begins with the drug situated above the pouch material. One roll of strip-pack material is used to form the pouch. This is accomplished by moving the material over a device that forces the material to fold into two equal sides. The sides and bottom are sealed prior to dosing. The strip pack may be cut later during the equipment processing or roll continuously and be manually cut. Temperature and dwell time are the main critical factors for this equipment.

Preformed Unit-Dose Packages

Preformed containers are sealed either by heat or by adhesion. Heat sealers may be manual units requiring hand pressure application or automated units that provide a more controlled pressure for sealing.

Heat sealing may be accomplished through the use of manual tabletop equipment, which is generally operated at a set pressure. Critical parameters with these devices are pressure and temperature control because undesirable variation in these parameters may yield inadequate seals.

Critical Parameters

In order to ensure that the finished container performs as intended, qualification of critical parameters should be determined. Typically, validation of a packaging line consists of qualification of the installation, operation, and performance of a packaging system.

INSTALLATION QUALIFICATION

Equipment should be installed and found to be in proper working condition prior to use.

OPERATIONAL QUALIFICATION

Operational qualification should be performed to establish that the equipment operates within the manufacturer's specified ranges. Incoming utilities for the equipment, such as air, electricity, etc., should be monitored and checked periodically.

PERFORMANCE QUALIFICATION

Performance qualification should be done to establish that the equipment performs properly with the required materials to produce a container that functions as intended. The critical parameters include forming temperature and pressure, sealing temperature and pressure, and dwell time at the seal station. Qualified ranges should be readily available in a reference source for the setup of equipment. Re-evaluation may be necessary with changes to equipment, materials, or process.

In-Process Inspections

Strict controls covering the packaging and labeling processes should be in place. The final container should be evaluated for performance in each of the stations previously described. Specifically, the formed container should be inspected visually to ensure that it is properly formed. Evaluation of the filling station should include a check to ensure that the unit dose is properly filled (i.e., that the correct product is present). The sealing station should be evaluated to ensure that a proper seal has been made and that the moisture permeation specifications of the sealed container have been met. A visual examination of the package should be performed to ensure that the final steps of the packaging process are acceptable.

Repackagers and dispensers should use a standard inspection plan to verify the adequacy of the package. A visual inspection should be performed to verify that the correct product is in the proper packaging materials with correct labeling. Seal integrity

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should be evaluated, using vacuum testing,[2] helium testing, tear testing, and other testing methods suitable to establish whether seal integrity is maintained.

PERFORMANCE

The primary purpose of the unit-dose package used in the packaging of a drug preparation is to ensure that, until its intended expiration date, there is adequate protection from the environment as the dosage form is distributed and stored. It is also essential that the materials used do not interact with the dosage form.

When determining what type of package to use in the repackaging operation, consideration must be given to the dosage form's sensitivities (if any) to the storage and distribution environments (e.g., temperature, light, and moisture).

The properties of the finished container are defined by the materials used in constructing the unit-dose container, and by the process used to form and seal the container. As discussed in *Materials*, there is a wide variety of commercially available film structures that provide unit-dose containers with a range of moisture and light protection. Suppliers of these materials typically provide quantitative data, obtained from well-established test methods, to highlight the protective properties of their material. These data are based on flat sheets of the film, not on the formed container.

It is critical to understand that once the film is formed, protective properties change because the overall thickness of the film decreases as the blister cavity is formed. Usually the change is a decrease, especially in the case of barrier properties. However, the extent of change will vary with the type of film structure used and is also highly dependent on the container-forming process used (see *Process*). Further, a suboptimal seal on the formed container will decrease the protective properties of the container. Insufficient temperature, time, or pressure during a heat-seal operation may enable the passage of moisture or oxygen through the seal area over time, which may have an effect on the dosage form. In addition, if the seal area is designed with insufficient surface area, the same problem may occur. To ensure a good seal, a minimum sealing distance of 3 mm from the edge of the blister cavity to the nearest edge or perforation is recommended. Therefore, it is important to measure the performance of the formed and sealed container rather than the performance of the flat sheet.

Moisture is a critical factor in preparation integrity. *Containers—Performance Testing* ⟨671⟩ describes how to determine and classify moisture permeation rates. If the manufacturer's labeling includes "Protect From Moisture," the repackager shall utilize a high barrier film.

If light protection is required for a drug preparation, the repackager should follow the requirements for light transmission established under *Containers—Performance Testing* ⟨671⟩. Again, this testing should be conducted on the formed container, because the light-protective properties of the film are compromised once the film is thinned during the forming process. It is recommended that these tests, in conjunction with any guidance provided by the manufacturer, be considered appropriate for any container–closure system used in repackaging a drug preparation.

BEYOND-USE-DATE

In the absence of stability data for the drug product in the repackaged container, the beyond-use dating period is one year or the time remaining until the expiration date, whichever is shorter. If current stability data are available for the drug product in the repackaged container, the length of time established by the stability study may be used to establish the beyond-use date, but must not exceed the manufacturer's expiration date.

The dispenser must maintain the facility where the dosage forms are packaged and stored at a temperature such that the mean kinetic temperature is not greater than 25°. The plastic material used in packaging the dosage forms must afford better protection than polyvinyl chloride, which does not provide adequate protection against moisture permeation. Records must be kept of the temperature of the facility where the dosage forms are stored, and of the plastic materials used in packaging.

MINIMUM REQUIREMENTS

The previous sections serve as a general introduction to repackaging by providing a basic understanding of materials selection, the form-fill-seal process, and the importance of performance of the sealed container. In this section, certain minimum requirements for repackaging, which must be met, are described in more detail.

Personnel

Each person with responsibility for the repackaging of a preparation shall have the education, training, and experience, or any combination thereof, to perform assigned functions in a manner such that the safety, identity, strength, quality, purity, potency, and pharmaceutical elegance of the drug dosage form are retained. Training should be documented.

Personnel engaged in the repackaging of a preparation shall wear clean clothing appropriate for the duties or processes performed.

[2] Vacuum testing consists of placing samples from the packaging operation into a jar filled with water. A lid is placed over the samples to fully immerse them in the water. A container lid is applied to create a seal effective enough to create approximately 25 cm of vacuum. The vacuum pump is set, and the samples are tested for approximately 1 minute, removed from the water, wiped down, and opened to determine whether the inside of the unit-dose cavity or pouch is wet. This process should be adjusted until it is under control, and additional testing may be performed to ensure that the seal integrity is consistently acceptable. Wetness indicates a defective seal and therefore the potential for the drug to degrade when exposed to the atmosphere. Defective packages must be removed from further use.

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Facility

The repackaging facility may require areas of low relative humidity, and temperature conditions should meet controlled room temperature requirements specified in *Packaging and Storage Requirements* ⟨659⟩.

Equipment

Equipment used in the repackaging of a preparation shall be of appropriate design and suitably located to facilitate operations for its intended use. Its design should allow for cleaning to preclude cross-contamination as well as for maintenance to be performed. Equipment shall be constructed so that those surfaces that contact components or a preparation are not reactive, additive, or absorptive. Any substances required for operation, such as lubricants or coolants, shall not come into contact with components or a preparation.

Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination. Preventive maintenance should be performed at appropriate intervals in accordance with the equipment manufacturer's recommendation. Any instruments used to monitor critical parameters should be calibrated on a defined schedule.

Process

Steps should be taken to determine the critical process parameters (e.g., seal temperature, dwell time) in operating the equipment. Set points for these parameters should be documented and procedures established to ensure that they are adhered to each time the equipment is operated.

Labeling

The labeling requirements for a commercial repackager and a pharmacist are different. For example, the commercial repackager must comply with 21 CFR 201.1, but the pharmacist or dispenser does not have to comply with this requirement. If stability data are unavailable, the dispenser shall repackage only an amount of stock sufficient for a limited time and shall include product name and strength, lot number, manufacturer, and appropriate beyond-use date on the label. When quantities are repackaged in advance of immediate needs, each preparation must bear an identifying label, and the dispenser is required to maintain suitable repackaging records showing the name of the manufacturer, lot number, expiration date, date of repackaging, and designation of persons responsible for repackaging and for checking. The repackager or dispenser will use documented controls to prevent labeling errors.

Materials

The repackager or dispenser shall place an appropriate beyond-use date on the label and package in appropriate materials. Materials used by the repackager shall not be reactive, additive, or absorptive, and must meet the requirements described in 21 CFR 175 and 177.

Storage

The dispenser shall rotate and monitor stock closely to ensure that the dispensing of preparations is on a first-in-first-out (FIFO) basis. The repackager or dispenser shall store preparations under required environmental conditions (e.g., controlled room temperature with a mean kinetic temperature not higher than 25°).

Drug Product

The repackager or dispenser shall examine preparations for evidence of instability such as change in color or odor, and shall exercise professional judgment as to the acceptability of a package.

Complaints

The repackager or dispenser will maintain written procedures describing the handling of written and oral complaints regarding a drug product and will ensure that complaints are investigated and appropriately resolved.

Returned Goods

Policies and procedures relating to returned goods should be developed to ensure proper handling.

Reprocessing

Reprocessing of repackaged unit-dose containers (i.e., removing medication from one unit-dose container and placing it into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister

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card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided the original beyond-use date is maintained, and provided the integrity of the blister is ensured.

Special Considerations

If a product is known to be oxygen sensitive or if it exhibits extreme moisture or light sensitivity (e.g., cold form foil), it shall not be repackaged. If a product is refrigerated, it shall not be repackaged unless proper environmental conditions and suitable materials are available. Certain drug products (such as oncologic agents, hormones, or penicillin derivatives) require special handling because they are considered very potent or toxic, and because transfer of any portion of these products to another product could have deleterious effects.

REPACKAGING NONSTERILE SOLID AND LIQUID DOSAGE FORMS INTO SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS

The following guidance is intended for those engaged in pharmaceutical dispensing, and does not apply to commercial dispensing. An official dosage form is required to bear on its label an expiration date assigned for the particular formulation and package of the article. This date limits the time during which the product may be dispensed or used. Because the expiration date stated on the original manufacturer's container–closure system has been determined for the drug in that particular system and is not intended to apply to a product that has been repackaged in a different container, repackaged drugs dispensed pursuant to a prescription are exempt from using the expiration date from the original manufacturer's package. However, under no circumstance should the repackaged pharmaceutical preparation's expiration date exceed the original manufacturer's expiration date. It is necessary, therefore, that other precautions be taken by the dispenser to preserve the strength, quality, and purity of drugs that are repackaged for ultimate distribution or sale to patients.

The following guidelines and requirements are applicable where official dosage forms are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

Labeling

It is the responsibility of the dispenser to place a suitable expiration date on the label, taking into account the nature of the drug repackaged, any packaging and expiration dating information in the manufacturer's product labeling, the characteristics of the containers, and the storage conditions to which the article may be subjected. Repackaged dosage forms must bear on their labels expiration dates as determined from information in the product labeling (see *Labeling* ⟨7⟩, *Expiration Date and Beyond-Use Date*). Each single-unit or unit-dose container bears a separate label, unless the device holding the unit-dose form does not allow for the removal or separation of the intact single-unit or unit-dose container therefrom.

Storage

Store the repackaged article in a humidity-controlled environment and at the temperature specified in the individual monograph or in the product labeling. For further directions, see *Packaging and Storage Requirements* ⟨659⟩.

A refrigerator or freezer shall not be considered to be a humidity-controlled environment. Drugs that are to be stored at a cold temperature in a refrigerator or freezer must be protected during storage in the refrigerator or freezer. An outer container may be necessary for such protection; it is recommended that the drug monograph be referenced for storage.

Reprocessing

Reprocessing of repackaged unit-dose containers (i.e., removing a dosage unit from one unit-dose container and placing it in another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided that the original expiration date is maintained.

CUSTOMIZED PATIENT MEDICATION PACKAGES

In lieu of dispensing two or more prescribed drug products in separate containers, a pharmacist may, with the consent of the patient, the patient's caregiver, or a prescriber, provide a customized patient medication package (patient med pak).[3]

A patient med pak, i.e., a package prepared by a pharmacist for a specific patient, comprises a series of containers and contains two or more prescribed solid oral dosage forms. The patient med pak is so designed, or each container is so labeled, as to indicate the day and time, or period of time, that the container contents are to be taken.

It is the responsibility of the dispenser to instruct the patient or caregiver on the use of the patient med pak.

[3] It should be noticed that for patient med paks there is no special exemption from the requirements of the Poison Prevention Packaging Act. Thus, the patient med pak, if it does not meet child-resistant standards, shall be placed in an outer package that does comply, or the necessary consent of the purchaser or physician to dispense in a container not intended to be child-resistant shall be obtained.

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Label

The patient med pak shall bear a label stating the following:

1. The name of the patient;
2. A serial number for the patient med pak itself and a separate identifying serial number for each of the prescription orders for each of the drug products contained therein;
3. The name, strength, physical description or identification, and total quantity of each drug product contained therein;
4. The directions for use and cautionary statements, if any, contained in the prescription order for each drug product therein;
5. Any storage instructions or cautionary statements required by the official compendia;
6. The name of the prescriber of each drug product;
7. The date of preparation of the patient med pak and the beyond-use date or period of time assigned to the patient med pak (such beyond-use date or period of time shall be not longer than the shortest recommended beyond-use date for any dosage form included therein or not longer than 60 days from the date of preparation of the patient med pak, and shall not exceed the shortest expiration date on the original manufacturer's bulk containers for the dosage forms included therein); alternatively, the package label shall state the date of the prescription(s) or the date of preparation of the patient med pak, provided the package is accompanied by a record indicating the start date and the beyond-use date;
8. The name, address, and telephone number of the dispenser (and the dispenser's registration number where necessary); and
9. Any other information, statements, or warnings required for any of the drug products contained therein.

If the patient med pak allows for the removal or separation of the intact containers therefrom, each individual container shall bear a label identifying each of the drug products contained therein.

Labeling

The patient med pak shall be accompanied by a patient package insert, in the event that any medication therein is required to be dispensed with such insert as accompanying labeling. Alternatively, such required information may be incorporated into a single, overall educational insert provided by the pharmacist for the total patient med pak.

Packaging

In the absence of more stringent packaging requirements for any of the drug products contained therein, each container of the patient med pak shall comply with the moisture permeation requirements for a Class B single-unit or unit-dose container (see *Containers—Performance Testing* ⟨671⟩). Each container shall be either nonreclosable or so designed as to show evidence of having been opened.

Guidelines

It is the responsibility of the dispenser, when preparing a patient med pak, to take into account any applicable compendial requirements or guidelines and the physical and chemical compatibility of the dosage forms placed within each container, as well as any therapeutic incompatibilities that may attend the simultaneous administration of the medications. In this regard, pharmacists are encouraged to report to USP headquarters any observed or reported incompatibilities. Once a medication has been placed in a patient med pak with another solid dosage form, it may not be returned to stock, redistributed, or resold if unused.

Recordkeeping

In addition to any individual prescription filing requirements, a record of each patient med pak shall be made and filed. Each record shall contain, as a minimum:

1. The name and address of the patient;
2. The serial number of the prescription order for each drug product contained therein;
3. The name of the manufacturer or labeler and lot number for each drug product contained therein;
4. Information identifying or describing the design, characteristics, or specifications of the patient med pak sufficient to allow subsequent preparation of an identical patient med pak for the patient;
5. The date of preparation of the patient med pak and the beyond-use date that was assigned;
6. Any special labeling instructions; and
7. The name or initials of the pharmacist who prepared the patient med pak.

GLOSSARY

Dispenser: A dispenser is a licensed or registered practitioner who is legally responsible for providing the patient with a preparation that is in compliance with a prescription or a medication order and contains a specific patient label. In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. Dispensers are governed by the board of pharmacy of the individual state.

Package: The term "package" is synonymous with the term "container". See *Packaging and Storage Requirements* ⟨659⟩.

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Pharmacy: A pharmacy is an establishment that is legally responsible for providing the patient with a drug preparation with a specific patient label, in compliance with a prescription or a medication order. The terms "dispenser" and "pharmacy" are used interchangeably.

Repackaging: Repackaging is the act of removing a preparation from its original primary container and placing it into another primary container, usually of smaller size.

Repackager: A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice regulations in 21 CFR 210 and 211.▲ (USP 1-May-2020)

⟨1151⟩ PHARMACEUTICAL DOSAGE FORMS

Change to read:

GENERAL CONSIDERATIONS

This chapter provides general descriptions of and definitions for drug products, or dosage forms, commonly used to administer the drug substance (active pharmaceutical ingredient; API). It discusses general principles involved in the manufacture or compounding of these dosage forms. A glossary is provided as a nomenclature resource and should be used in conjunction with the *Nomenclature Guidelines*.[1]

A dosage form is a pharmaceutical preparation consisting of drug substance(s) and/or excipient(s) to facilitate dosing, administration, and delivery of the content of the drug product or placebo to the patient. The design, materials, manufacturing, and testing of all dosage forms target drug product quality. A testing protocol must consider not only the physical, chemical, microbiological, and biological properties of the dosage form as appropriate, but also the administration route and desired dosing regimen. These considerations, organized by route of administration, are detailed in general chapters *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests* ⟨1⟩, *Oral Drug Products—Product Quality Tests* ⟨2⟩, *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩, *Mucosal Drug Products—Product Quality Tests* ⟨4⟩, *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* ⟨5⟩, and *Ophthalmic Products—Quality Tests* ⟨771⟩.[2] The organization of this general information chapter is by the quality attributes of each particular dosage form, generally without specific reference to the route of administration. The list below provides the preferred dosage forms used in official article titles. In addition to the preferred dosage forms, the *Glossary* contains other terms that have been used in current official article titles but are not preferred and should not be used for new drug product titles.

Official Dosage Forms Used in Official Article Titles

- Aerosols
- Capsules
- Creams
- Emulsions
- Films
- Foams
- Gases
- Gels
- Granules
- Gums
- Implants
- Injections
- Inserts
- Irrigations
- Liquids
- Lotions
- Lozenges
- Ointments
- Pastes
- Pellets
- Pills
- Powders
- Rinses
- Shampoos
- Soaps
- Solutions
- Sprays
- Strips
- Suppositories
- Suspensions
- Systems
- Tablets

Tests to ensure compliance with *USP* standards for dosage form performance fall into one of the following areas.

Dose Uniformity

(See also *Uniformity of Dosage Units* ⟨905⟩.) Consistency in dosing for a patient or consumer requires that the variation in the drug substance content of each dosage unit be accurately controlled throughout the manufactured batch or compounded lot of drug product. Uniformity of dosage units typically is demonstrated by one of two procedures: content uniformity or weight variation. The procedure for content uniformity requires the appropriate assay of the drug substance content of individual units. The procedure for weight variation uses the weight of the individual units to estimate their content. Weight variation may be used where the underlying distribution of the drug substance in the blend is presumed to be uniform and well-controlled, as in solutions. In such cases, the content of the drug substance may be adequately estimated by the net weight. Content uniformity does not rely on the assumption of blend uniformity and can be applied in all cases. Successful development and manufacture of dosage forms requires careful evaluation of the drug substance particle or droplet size, incorporation techniques, and excipient properties.

[1] *Nomenclature Guidelines*, http://www.usp.org/health-quality-safety/compendial-nomenclature.

[2] Marshall K, Foster TS, Carlin HS, & Williams RL. Development of a compendial taxonomy and glossary for pharmaceutical dosage forms. *Pharm Forum.* 2003;29(5):1742–1752.

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Stability

Drug product stability involves the evaluation of chemical stability, physical stability, and performance over time. The chemical stability of the drug substance in the dosage form matrix must support the expiration dating for the commercially prepared dosage forms and a beyond-use date for a compounded dosage form. Test procedures for potency must be stability indicating (see *Validation of Compendial Procedures* ⟨1225⟩). Degradation products should be quantified. In the case of dispersed or emulsified systems, consideration must be given to the potential for settling or separation of the formulation components. Any physical changes to the dosage form must be easily reversed (e.g., by shaking) prior to dosing or administration. For tablets, capsules, oral suspensions, and implants, in vitro release test procedures such as dissolution and disintegration provide a measure of continuing consistency in performance over time (see *Dissolution* ⟨711⟩, *Disintegration* ⟨701⟩, and *Drug Release* ⟨724⟩).

Bioavailability

(See also *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩ and *Assessment of Solid Oral Drug Product Performance and Interchangeability, Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩.) Bioavailability is influenced by factors such as the method of manufacture or compounding, particle size, crystal form (polymorph) of the drug substance, the properties of the excipients used to formulate the dosage form, and physical changes as the drug product ages. Assurance of consistency in bioavailability over time (bioequivalence) requires close attention to all aspects of the production (or compounding) and testing of the dosage form. With proper justification, in vitro release testing (e.g., disintegration and dissolution) may be used as a surrogate to demonstrate consistent availability of the drug substance from the formulated dosage.

Release Profile

Two principal categories of drug release are recognized: immediate-release and modified-release.

"Immediate-release" is observed when no deliberate effort has been made to modify the drug substance release profile. For example, capsules and tablets are considered immediate-release even if a disintegrating agent or a lubricant has been used.

"Modified-release" is a term used when the rate and/or time of release of the drug substance is altered as compared to what would be observed or anticipated for an immediate-release product. Two modified-release profiles, delayed-release and extended-release, are recognized. The term "modified-release" is not used for official article titles.

"Delayed-release" is used when deliberate formulation achieves a delay in the release of the drug substance for some period of time after initial administration. For oral products, expressions such as "enteric-coated" or "gastro-resistant" have also been used where release of the drug substance is prevented in the gastric environment but promoted in the intestinal environment. However, the term "delayed-release" is used for official article titles.

"Extended-release" is used when the deliberate formulation achieves prolongation of drug substance release compared to that observed or anticipated for an immediate-release dosage form. Expressions such as "prolonged-release", "repeat-action", "controlled-release", "long-acting", and "sustained-release" have also been used to describe such dosage forms. However, the term "extended-release" is used for official article titles.

The *Nomenclature Guidelines*[1] should be consulted for naming conventions for products with a single drug substance or for products with a combination of more than one drug substance displaying the combination of release profiles of immediate-release and extended-release, immediate-release and delayed-release, or extended-release and delayed-release.

Manufacture

Although detailed instructions about the manufacture of any of these dosage forms are beyond the scope of this general information chapter, general manufacturing principles have been included.[3] Information relative to extemporaneous compounding of dosage forms can be found in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩.

Route of Administration

The primary routes of administration for pharmaceutical dosage forms can be defined as parenteral (see ⟨1⟩), gastrointestinal (see ⟨2⟩), topical (see ⟨3⟩), mucosal, and (see ⟨4⟩), inhalation (see ⟨5⟩). Each has subcategories as needed. Many tests used to ensure quality generally are applied across all of the administration routes, but some tests are specific for individual routes. For example, products intended for injection must be evaluated using *Sterility Tests* ⟨71⟩, *Bacterial Endotoxins Test* ⟨85⟩, or *Pyrogen Test* ⟨151⟩, and the manufacturing process (and sterilization technique) employed for parenterals (by injection) should ensure compliance with these tests. Tests for particulate matter may be required for certain dosage forms depending on the route of administration (e.g., by injection—*Particulate Matter in Injections* ⟨788⟩, or mucosal—*Particulate Matter in Ophthalmic Solutions* ⟨789⟩). Additionally, dosage forms intended for the inhalation route of administration must be monitored for particle size and spray pattern (for a metered-dose inhaler or dry powder inhaler) and droplet size (for nasal sprays). Further information regarding administration routes and suggested testing can be found in the *Guide to General Chapters, Chapter Charts, Charts 4–8, 10,* and *13*.

An appropriate manufacturing process and testing regimen helps ensure that a dosage form can meet the appropriate quality attributes for the intended route of administration.

[3] The terms "manufacture" and "preparation" are used interchangeably in this general chapter.

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Packaging and Storage

Suitable packaging is determined for each product. For additional information about meeting packaging requirements listed in the individual labeling, refer to *Packaging and Storage Requirements* ⟨659⟩, *Containers—Performance Testing* ⟨671⟩, ▲▲(CN 1-May-2020)and *Good Repackaging Practices* ⟨1178⟩. Product labeling must specify storage requirements that describe environmental conditions, limitations, and restrictions. For instance, exposure to excessive temperature, humidity, and light can influence the ability of the packaging to protect the product.

Labeling Statements

Some dosage forms or articles have mandatory labeling statements that are given in the *Code of Federal Regulations* (e.g., 21 CFR §201.320 and 21 CFR §369.21). The text of 21 CFR should be consulted to determine the current recommendations.

Change to read:

PRODUCT QUALITY TESTS, GENERAL

International Council for Harmonisation (ICH) Guidance Q6A (available at www.ich.org) recommends specifications (list of tests, references to analytical procedures, and acceptance criteria) to ensure that drug products are safe and effective at the time of release and over their shelf life. Tests that are universally applied to ensure safety, efficacy, strength, quality, and purity include description, identification, assay, and impurities.

Description

The *Definition* section (see *General Notices, 4.10 Monographs*) in a *USP* monograph describes the drug product and specifies the range of acceptable assayed content of the drug substance(s) present in the dosage form. For certain products, the *Definition* includes any relevant additional information, such as the presence or absence of other components, excipients, or adjuvants, cautionary statements on toxicity and stability, etc. While appearance information to aid in identification is used in a regulatory submission (e.g., a qualitative description of size, shape, color, etc.) it is typically not required as part of a *USP* monograph. This information is drug product specific.

Identification

Identification tests are discussed in *General Notices, 5.40 Identification*. Identification tests should establish the identity of the drug substance(s) present in the drug product and should discriminate between compounds of closely related structure that are likely to be present. Identification tests should be specific for the drug substance(s). For example, the infrared absorption spectrum is often used (see *Mid-Infrared Spectroscopy* ⟨854⟩ and ▲*Spectroscopic Identification Tests* ⟨197⟩▲(CN 1-May-2020)). If no suitable infrared spectrum can be obtained, other analytical methods can be used. Near-infrared (NIR) or Raman spectrophotometric methods could also be acceptable as the sole identification method of the drug product formulation (see ▲*Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩▲(CN 1-May-2020) and *Raman Spectroscopy* ⟨1120⟩). Identification by a chromatographic retention time from a single procedure is not regarded as specific. The use of retention times from two chromatographic procedures for which the separation is based on different principles or a combination of tests in a single procedure can be acceptable (see *Chromatography* ⟨621⟩ and *Thin-Layer Chromatographic Identification Test* ⟨201⟩).

Assay

A specific and stability-indicating test should be used to determine the strength (drug substance content) of the drug product. Some examples of these procedures are *Antibiotics—Microbial Assays* ⟨81⟩, ⟨621⟩, or *Assay for Steroids* ⟨351⟩. In cases when the use of a nonspecific assay is justified (e.g., *Titrimetry* ⟨541⟩), other supporting analytical procedures should be used to achieve specificity. When evidence of excipient interference with a nonspecific assay exists, a procedure with demonstrated specificity should be used.

Impurities

Process impurities, synthetic byproducts, and other inorganic and organic impurities may be present in the drug substance and excipients used in the manufacture of the drug product. These impurities are evaluated by tests in the drug substance and excipients monographs. Impurities arising from degradation of the drug substance or from the drug-product manufacturing process should be monitored. *Residual Solvents* ⟨467⟩ is applied to all products where relevant.

In some cases, testing for heavy metal impurities is appropriate.

In addition to the universal tests listed, the following tests may be considered on a case-by-case basis.

Physicochemical Properties

Examples include *pH* ⟨791⟩, *Viscosity—Capillary Methods* ⟨911⟩ or *Viscosity—Rotational Methods* ⟨912⟩, and *Specific Gravity* ⟨841⟩.

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Particle Size

For some dosage forms, particle size can have a significant effect on dissolution rates, bioavailability, therapeutic outcome, and stability. Procedures such as those found in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ and *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩ could be used.

Uniformity of Dosage Units

See the discussion of *Dose Uniformity* in the *General Considerations* section.

Water Content

A test for water content is included when appropriate (see *Water Determination* ⟨921⟩).

Microbial Limits

The type of microbial test(s) and acceptance criteria are based on the nature of the nonsterile drug product, method of manufacture, and the route of administration (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩).

Antimicrobial Preservative Content

Acceptance criteria for preservative content in multidose products should be established. They are based on the levels of antimicrobial preservative necessary to maintain the product's microbiological quality at all stages throughout its proposed usage and shelf life (see *Antimicrobial Effectiveness Testing* ⟨51⟩).

Antioxidant Content

If antioxidants are present in the drug product, tests of their content should be performed to maintain the product's quality at all stages throughout its proposed usage and shelf life.

Sterility

Depending on the route of administration (e.g., ophthalmic preparations, implants, aqueous-based preparations for oral inhalation, and injections) sterility of the product is demonstrated as appropriate (see ⟨71⟩).

Dissolution

A test to measure the release of the drug substance(s) from the drug product normally is included for dosage forms such as tablets, capsules, suspensions, granules for suspensions, implants, transdermal delivery systems, and medicated chewing gums. Single-point measurements typically are used for immediate-release dosage forms. For modified-release dosage forms, appropriate test conditions and sampling procedures are established as needed (see ⟨711⟩ and ⟨724⟩). In some cases, dissolution testing may be replaced by disintegration testing (see ⟨701⟩).

Breaking Force and Friability

These parameters are evaluated as in-process controls. Acceptance criteria depend on packaging, supply chain, and intended use (see *Tablet Friability* ⟨1216⟩ and *Tablet Breaking Force* ⟨1217⟩).

Leachables

When evidence exists that leachables from the container–closure systems (e.g., rubber stopper, cap liner, or plastic bottle) have an impact on the safety or efficacy of the drug product, a test is included to evaluate the presence of leachables.

Other Tests

Depending on the type and composition of the dosage form, other tests such as alcohol content, redispersibility, particle size distribution, rheological properties, reconstitution time, endotoxins/pyrogens, particulate matter, functionality testing of delivery systems, delivered dose uniformity, viscosity, and osmolarity may be necessary.

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DOSAGE FORMS

Aerosols

Aerosols are dosage forms packaged under pressure and contain therapeutic agent(s) and a propellant that are released upon actuation of an appropriate valve system. Upon actuation of the valve system, the drug substance is released as a plume of fine particles or droplets. Only 1 dose is released from the preparation upon actuation of a metered valve. In the case of topical products and depending on the nature of the drug substance and the conditions being treated, actuation of the valve may result in a metered release of a controlled amount of the formulation or the continuous release of the formulation as long as the valve is depressed.

The aerosol dosage form refers only to those products packaged under pressure that release a fine mist of particles or droplets when actuated (see *Glossary*). Other products that produce dispersions of fine droplets or particles will be covered in subsequent sections (e.g., *Powders* and *Sprays*).

TYPICAL COMPONENTS

Typical components of aerosols are the formulation containing one or more drug substance(s) and propellant, the container, the valve, and the actuator. Each component plays a role in determining various characteristics of the emitted plume, such as droplet or particle size distribution, uniformity of delivery of the therapeutic agent, delivery rate, and plume velocity and geometry. The metering valve and actuator act in tandem to generate the plume of droplets or particles. The metering valve delivers an accurate volume of the pressurized liquid formulation from the container. The actuator directs the metered volume to a small orifice that is open to the atmosphere. Upon actuation, the formulation is forced through the opening, forming the fine mist of particles that are directed to the site of administration.

Aerosol preparations may consist of either a two-phase (gas and liquid) or a three-phase (gas, liquid, and solid or liquid) formulation. The two-phase formulation consists of drug substance(s) dissolved in liquefied propellant. Co-solvents such as alcohol may be added to enhance the solubility of the drug substance(s). Three-phase inhalation and nasal aerosol systems consist of suspended drug substance(s) in propellant(s), co-solvents, and potentially other suitable excipients. The suspension or emulsion of the finely divided drug substance is typically dispersed in the liquid propellant with the aid of suitable biocompatible surfactants or other excipients.

Propellants for aerosol formulations are typically low molecular weight hydrofluorocarbons or hydrocarbons that are liquid when constrained in the container, exhibit a suitable vapor pressure at room temperature, and are biocompatible and nonirritating. Compressed gases do not supply a constant pressure over use and typically are not used as propellants.

Metal containers can withstand the vapor pressure produced by the propellant. Excess formulation may be added to the container to ensure that the full number of labeled doses can be accurately administered. The container and closure must be able to withstand the pressures anticipated under normal use conditions as well as when the system is exposed to elevated temperatures.

TYPES OF AEROSOL DOSAGE FORMS

Aerosol dosage forms can be delivered via various routes. The container, actuator, and metering valve, as well as the formulation, are designed to target the site of administration.

Inhalation aerosols, commonly known as metered-dose inhalers (MDIs), are intended to produce fine particles or droplets for inhalation through the mouth and deposition in the pulmonary tree. The design of the delivery system is intended to release measured mass and appropriate quality of the active substance with each actuation.

Nasal aerosols, commonly known as nasal MDIs, produce fine particles or droplets for delivery through the nasal vestibule and deposition in the nasal cavity. Each actuation of the valve releases a measured mass of the drug substance with appropriate quality characteristics.

Lingual aerosols are intended to produce fine particles or droplets for deposition on the surface of the tongue. The design of the delivery system releases 1 dose with each actuation.

Topical aerosols produce fine particles or droplets for application to the skin.

LABELING FOR PROPER USE

Refer to 21 CFR §201.320 and 21 CFR §369.21.

Capsules

Capsules are solid dosage forms in which the drug substance and/or excipients are enclosed within a soluble container or shell or coated on the capsule shell. The shells may be composed of two pieces (a body and a cap), or they may be composed of a single piece. Two-piece capsules are commonly referred to as hard-shell capsules, and one-piece capsules are often referred to as soft-shell capsules. This two-piece and one-piece capsule distinction, although imprecise, reflects differing levels of plasticizers in the two compositions and the fact that one-piece capsules typically are more pliable than two-piece capsules.

The shells of capsules are usually made from gelatin. However, they may also be made from cellulose polymers or other suitable material. Most capsules are designed for oral administration. When no deliberate effort has been made to modify the drug substance release rate, capsules are referred to as immediate-release.

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TWO-PIECE OR HARD-SHELL CAPSULES

Two-piece capsules consist of two telescoping cap and body pieces in a range of standard sizes.

ONE-PIECE OR SOFT-SHELL CAPSULES

One-piece capsules typically are used to deliver a drug substance as a solution or suspension. Liquid formulations placed into one-piece capsules may offer advantages by comparison with dry-filled capsules and tablets in achieving content uniformity of potent drug substance(s) or acceptable dissolution of drug substance(s) with poor aqueous solubility. Because the contact between the shell wall and its liquid contents is more intimate than in dry-filled capsules, undesired interactions may be more likely to occur (including gelatin crosslinking and pellicle formation).

MODIFIED-RELEASE CAPSULES

The release of drug substance(s) from capsules can be modified in several ways. There are two categories of modified-release capsule formulations recognized by USP.

Delayed-release capsules: Capsules are sometimes formulated to include enteric-coated granules to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation. Enteric-coated multiparticulate capsule dosage forms may reduce variability in bioavailability associated with gastric emptying times for larger particles (i.e., tablets) and to minimize the likelihood of a therapeutic failure when coating defects occur during manufacturing. Alternatively, a coating may be applied to the capsule shell to achieve delayed release of the contents.

Extended-release capsules: Extended-release capsules are formulated in such a manner as to make the contained drug substance available over an extended period of time following ingestion. Requirements for dissolution (see ⟨711⟩) are typically specified in the individual monograph.

Methods for modifying drug substance release from capsules include coating the filled capsule shells or the contents, in the case of dry-filled capsules.

PREPARATION

Two-piece capsules: Two-piece gelatin capsules are usually formed from blends of gelatins that have relatively high gel strength in order to optimize shell clarity and toughness or from hypromellose. They may also contain colorants such as Drug & Cosmetic (D&C) and Food, Drug, & Cosmetic (FD&C) dyes[4] or various pigments, opaquing agents such as titanium dioxide, dispersing agents, plasticizers, and preservatives. Gelatin capsule shells normally contain between 12% and 16% water.

The shells are manufactured in one set of operations and later filled in a separate manufacturing process. Two-piece shell capsules are made by a process that involves dipping shaped pins into gelatin or hypromellose solutions, followed by drying, cutting, and joining steps.

Powder formulations for two-piece gelatin capsules generally consist of the drug substance and at least one excipient. Both the formulation and the method of filling can affect release of the drug substance. In the filling operation, the body and cap of the shell are separated before filling. Following the filling operation, the machinery rejoins the body and cap and ensures satisfactory closure of the capsule by exerting appropriate force on the two pieces. The joined capsules can be sealed after filling by a band at the joint of the body and cap or by a designed locking joint between the cap and body. In compounding prescription practice, two-piece capsules may be hand-filled. This permits the prescriber the choice of selecting either a single drug substance or a combination of drug substances at the exact dose level considered best for an individual patient.

One-piece capsules: One-piece capsules are formed, filled, and sealed in a single process on the same machine and are available in a wide variety of sizes, shapes, and colors. The most common type of one-piece capsule is that produced by a rotary die process that results in a capsule with a seam. The soft gelatin shell is somewhat thicker than that of two-piece capsules and is plasticized by the addition of polyols such as glycerin, sorbitol, or other suitable materials. The ratio of the plasticizer to the gelatin can be varied to change the flexibility of the shell depending on the nature of the fill material, its intended usage, or environmental conditions.

In most cases, one-piece capsules are filled with liquids. Typically, drug substances are dissolved or suspended in a liquid vehicle. Classically, an oleaginous vehicle such as a vegetable oil was used. However, nonaqueous, water-miscible liquid vehicles such as the lower molecular weight polyethylene glycols are now more common. The physicochemical properties of the vehicle can be chosen to ensure stability of the drug substance as well as to influence the release profile from the capsule shell.

Creams

(See *Emulsions*.)

[4] In 1960 Congress enacted the Color Additive Amendments, requiring the FDA to regulate dyes, pigments, or other coloring agents in foods, drugs, and cosmetics separately from food additives. Under the law, color additives are deemed unsafe unless they are used in compliance with FDA regulations. The law provides a framework for the listing and certification of color additives. See FDCA §721; see FDA regulations at 21 CFR Part 70. Colors must also be listed in pertinent FDA regulations for specific uses; the list of color additives for drugs that are exempt from certification is published at 21 CFR Part 73, Subpart B. FDA also conducts a certification program for batches of color additives that are required to be certified before sale; see 21 CFR Part 74 (Subpart B re: drugs). Regulations regarding certification procedures, general specifications, and the listing of certified provisionally listed colors are at 21 CFR Part 80. FDA maintains a color additives website with links to various legal and regulatory resources at: http://www.fda.gov; search by document title.

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Emulsions

An emulsion is a dispersed colloidal system consisting of two immiscible liquid phases generally stabilized with one or more suitable agents.

Typical pharmaceutical emulsions are prepared from immiscible aqueous and organic (oil) liquids. Complex multiple-phase systems may exist in an emulsion. Whether the organic or the aqueous phase is the dispersed phase depends on the volumes of the two phases, the emulsifier chosen, and the method of preparation. When an oil phase is dispersed in an aqueous phase, the emulsion is termed an oil-in-water (O/W) emulsion and water is referred to as the continuous phase. When water is dispersed in oil, the emulsion is referred to as a water-in-oil (W/O) emulsion. Emulsions have dispersed phases typically ranging from 0.1 to 100 µm. Emulsions are opaque while microemulsions are usually transparent or translucent. Microemulsions have dispersed phases less than 0.1 µm.

Emulsions may exhibit three types of instability: flocculation, creaming, and coalescence. Flocculation describes the process by which the dispersed phase comes out of suspension in the form of flakes. Coalescence is another form of instability—small droplets within the media continuously combine to form progressively larger droplets. Emulsions can also undergo creaming, where one of the phases migrates to the top (or the bottom, depending on the relative densities of the two phases) of the emulsion. To prevent flocculation, creaming, and coalescence of the emulsions, manufacturers commonly add surfactants, pH-modifying agents, or emulsifying agents to increase the stability of emulsions so that the emulsion does not change significantly with time.

Emulsions are widely used as pharmaceutical dosage forms. Oral emulsions have been prepared to improve taste, solubility, stability, or bioavailability. Emulsions for topical administration are referred to as creams, lotions, and sometimes ointments. Parenteral emulsions have been used for anesthetics, parenteral nutrition, and to deliver poorly water-soluble drugs.

CREAMS

Creams are semisolid emulsion dosage forms. They often contain more than 20% water and volatiles, and/or typically contain less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or to the mucous membranes. Creams have a relatively soft, spreadable consistency and can be formulated as either a W/O emulsion (e.g., *Cold Cream* or *Fatty Cream* as in the *European Pharmacopoeia*) or as an oil-in-water emulsion (e.g., *Betamethasone Valerate Cream*). Creams are generally described as either nonwashable or washable, reflecting the fact that an emulsion with an aqueous external continuous phase is more easily removed than one with a nonaqueous external phase (W/O emulsion).

LOTIONS

Lotions are an emulsified liquid dosage form intended for external application to the skin. Historically, some topical suspensions such as calamine lotion have been called lotions but that nomenclature is not currently preferred. Lotions share many characteristics with creams. The distinguishing factor is that they are more fluid than semisolid and thus pourable. Due to their fluid character, lotions are more easily applied to large skin surfaces than semisolid preparations. Lotions may contain antimicrobial agents as preservatives.

INJECTABLE EMULSIONS

Injectable emulsions are sterile liquid dosage forms of drug substances dissolved or dispersed in a suitable emulsion medium. Injectable emulsions are for parenteral administration of poorly water-soluble drugs.

OINTMENTS

Ointments are sometimes semisolid emulsion dosage forms (see *Dosage Forms, Ointments*).

PREPARATION

Chapter ⟨795⟩ provides general information regarding the preparation of emulsions.

Creams: Creams may be formulated from a variety of oils, both mineral and vegetable, and from fatty alcohols, fatty acids, and fatty esters. Emulsifying agents include nonionic surfactants, detergents, and soaps. Soaps are usually formed in situ during the preparation of creams from a fatty acid in the oil phase hydrolyzed by a base dissolved in the aqueous phase.

Preparation usually involves separating the formula components into two portions: lipid and aqueous. The lipid portion contains all water-insoluble components and the aqueous portion contains the water-soluble components. Both phases are heated to a temperature above the melting point of the highest melting component. The phases are then mixed and the mixture is stirred until reaching ambient temperature or until the mixture has congealed. Mixing is generally continued during the cooling process to promote uniformity. Traditionally, the aqueous phase is added to the lipid phase, but comparable results have been obtained with the reverse procedure. High-shear homogenization may be employed to reduce particle or droplet size and to improve the physical stability of the resultant dosage form.

The drug substance(s) can be added to the phase in which it is soluble at the beginning of the manufacturing process, or it can be added after the cream is prepared by a suitable dispersion process such as levigation or milling with a roller mill. Creams usually require the addition of a preservative(s) unless they are compounded immediately prior to use and intended to be consumed in a relatively short period of time.

Lotions: Lotions are usually prepared by dissolving or dispersing the drug substance into the more appropriate phase (oil or water), adding the appropriate emulsifying or suspending agents, and mixing the oil and water phases to form a uniform fluid emulsion.

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Injectable emulsions: Chapter ⟨1⟩ provides guidance on sterile preparations. Emulsions intended for parenteral administration can be formulated using the same principles as creams and lotions. The formulation should be designed for ease of administration. The particle size of the dispersed phase can vary by route of administration. For example, emulsions intended for intravenous administration should comply with *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩. The procedure to assure sterility should be validated by media fills. Preservatives are generally not used in injectable emulsions.
Ointments: (See *Dosage Forms, Ointments*.)

Films

Films are thin sheets that are placed in the oral cavity. They contain one or more layers. A layer may or may not contain the drug substance. Typically, these thin sheets are formed by casting or extrusion that results in a dispersion of the components through the film. Films are classified by the site of application. "Oral films" can be formulated to deliver medication to the mouth such as oral hygiene products or to deliver medication to the gastrointestinal tract for absorption. "Buccal films" and "sublingual films" are formulated to facilitate absorption through the proximal mucosal membranes avoiding first pass metabolism or degradation in the gastrointestinal tract and providing a quick onset of action.

Films can be formulated with edible polymers such as pullulan or with water-soluble polymers such as modified cellulose, edible gums, and copolymers. The dissolution rate of the film is controlled to facilitate incorporation of the medication into saliva or for absorption by the proximal mucosa. These films must be substantial enough to maintain their integrity during manufacture and packaging, and permit handling by the patient. Because of the rapid dissolution, taste and mouth feel are important considerations.

Foams

Foams are dispersions of gas in a liquid or solid continuous phase wherein the liquid or solid contains the drug substance and suitable excipients. Typical excipients intended for foam dosage forms include surfactants to ensure distribution of the gas/propellant in the formulation, aqueous or nonaqueous vehicles, and propellants (for pressurized systems). Foams are produced by mechanical means or via interaction of propellant gas and the formulation under pressure. Foams dispensed from nonpressurized containers use mechanical force to mix the formulation and air resulting in foam generation. Foams dispensed from pressurized containers use the propellant present in the gas phase to increase pressure inside the container. When the nozzle of the actuator is opened, the liquid phase is pushed out through specific actuators resulting in foam generation.

Medicated foams intended to treat severely injured skin or open wounds must be sterile.

PREPARATION

A foam may contain one or more drug substances, surfactants, and aqueous or nonaqueous liquids, and is produced with or without the aid of propellants. When a propellant is not used, mechanical work is required to generate the foam. If the propellant is in the internal (discontinuous) phase, a stable foam is discharged. If the propellant is in the external (continuous) phase, a quick-breaking foam is discharged.

Gases

Medical gases are products that are administered directly as a gas. A medical gas has a direct pharmacological action or acts as a diluent for another medical gas. Gases used as excipients for administration of aerosol products, as adjuvants in packaging, or produced by other dosage forms, are not included in this definition.

COMPONENTS

Medical gases may be single components or defined mixtures of components. Mixtures can also be extemporaneously prepared at the point of use.

ADMINISTRATION

Medical gases may be administered to the patient using several methods: nasal cannulas, face masks, atmospheric tents, and endotracheal tubes for the pulmonary route; hyperbaric chambers for the pulmonary and topical routes of administration; jetted tubes that are directed at dental tissue to promote drying in preparation for fillings and crowns; tubes for expanding the intestines to facilitate medical imaging during colonoscopy; tubes for expanding the pelvis via transuterine inflation in preparation for fallopian tubal ligation; and tubes for expanding angioplasty devices. The dose of medical gas is typically metered by a volume rate of flow under ambient temperature and pressure conditions. Administration of a highly compressed gas generally requires a regulator to decrease the pressure, a variable-volume flow controller, and suitable tubing to conduct the gas to the patient. For pulmonary administration, the gas flow will be directed to the nose or mouth by a suitable device or into the trachea through a mechanical ventilator. When medical gases are administered chronically, provision for humidification is common. Care should be exercised to avoid microbial contamination.

SPECIAL CONSIDERATIONS

The container and system fittings should be appropriate for the medical gas. Adaptors should not be used to connect containers to patient-use supply system piping or equipment. Large quantities of gases such as oxygen or nitrogen can be stored in the liquid state in a cryogenic container and converted into a gas, as needed, by evaporation. Additional rules

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concerning the construction and use of cryogenic containers are promulgated by governmental agencies (e.g., U.S. Department of Commerce).

Containers, tubing, and administration masks employed for gases containing oxygen are free of any compound that would be sensitive to oxidation or that would be irritating to the respiratory tract.

A significant fraction of the dose of a medical gas may be released into the general vicinity of the patient due to incomplete absorption. Adequate ventilation may be necessary to protect health care workers and others from exposure to the gas (e.g., nitrous oxide).

Gels

A gel is solid or semisolid. Gels can be classified in two groups, chemical and physical gels. Chemical gels are usually covalently crosslinked gels, while physical gels consist of small molecules or molecular chains that are physically crosslinked into networks, or solutions, or colloidal dispersions that are stiffened by a gelling agent. Typically, gels hold their form being self-supporting. Some gels may exhibit a range of behavior under mechanical forces. Gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation. Like emulsions, gels can be characterized as having a continuous phase as well as a dispersed phase. A variety of routes are available for gel administration such as topical, mucosal, or oral. In veterinary medicine, gels can also be administered via mammary infusion.

Gels may consist of a network of small discrete particles (e.g., *Aluminum Hydroxide Gel*, *Bentonite Magma*, or *Psyllium Hemicellulose*). As these gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation, they should be shaken before use to ensure homogeneity and should be so labeled.

Gels can consist of organic macromolecules uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid continuous phase. These gels may be made from natural or synthetic macromolecules (e.g., carbomer, hypromellose, or starch) or natural gums (e.g., tragacanth). Although these gels are commonly aqueous based, alcohols and oils may be used as the continuous phase.

Chewable gels are used to deliver drug substances or dietary supplements via the oral route. In addition to drug substances(s) or dietary supplements, chewable gels can consist of all or some of the following components—gelling agent(s), sugars, water, sweeteners, and flavoring agents. The sweeteners and flavoring are intended to enhance patient acceptance and mask the taste of the delivered labeled drug substance or dietary supplement. Chewable gels maintain their molded shape, are elastic, and yield to mastication. They are intended to be chewed before swallowing. Chewable gels are also known as "gummies" in the confectionary and dietary supplement industries but that term is not used in official article titles.

PREPARATION

Gels may be formed by dispersing the gelling agent in the continuous phase (e.g., by heating starch), by crosslinking the dispersed phase gelling agent, by changing the pH (as for *Carbomer Copolymer*), or by reducing the continuous phase by heat or vacuum (as for gels formed with sucrose).

Care should be taken to ensure uniformity of the drug substances by dispersing them by vigorous mixing or milling, or by shaking if the preparation is less viscous.

Chewable gels are formulated with one or more gelling agents (such as gelatin or starch), sugars (such as sucrose, fructose, or corn syrup), flavoring agents, sweeteners, colorants, and water. The ingredients are blended and heated to form a viscous solution that is poured into molds (e.g., corn starch molds). After cooling, the individual units are separated from the molds.

Granules

Granules are solid dosage forms that are composed of agglomerations of smaller particles. These multicomponent compositions are prepared for oral administration and are used to facilitate flexible dosing regimens as granules or as suspensions, address stability challenges, allow taste masking, or facilitate flexibility in administration (for instance, to pediatric patients, geriatric patients, or animals). Granular dosage forms may be formulated for direct oral administration and may facilitate compounding of multiple drug substances by allowing compounding pharmacists to blend various granular compositions in the retail or hospital pharmacy. More commonly, granules are reconstituted to a suspension by the addition of water or a supplied liquid diluent immediately prior to delivery to the patient. Effervescent granules are formulated to liberate gas (carbon dioxide) upon addition of water. Common examples of effervescent granules include antacid and potassium supplementation preparations. Common therapeutic classes formulated as granule dosage forms include antibiotics, certain laxatives (such as senna extract products), electrolytes, and various cough and cold remedies that contain multiple drug substances.

PREPARATION

Granules are often the precursors used in tablet compression or capsule filling. Although this application represents a pharmaceutical intermediate and not a final dosage form, numerous commercial products are based on granules. In the typical manufacture of granules, the drug substance(s) is blended with excipients (processing aids) and wetted with an appropriate pharmaceutical binding solution, solvent, or blend of solvents to promote agglomeration. This composition is dried and sized to yield the desired material properties.

Frequently, granules are used because the drug substance is unstable in aqueous environments and cannot be exposed to water for periods sufficient to accommodate manufacture, storage, and distribution in a suspension. Preparation of the liquid dosage form from the granules immediately prior to dispensing allows acceptable stability for the duration of use. Granules manufactured for this purpose are packaged in quantities sufficient for a limited time period—usually one course of therapy that typically does not exceed 2 weeks. In addition to the drug substances, other ingredients may be added to ensure acceptable

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stability (e.g., buffers, antioxidants, or chelating agents) or to provide color, sweetness, and flavor; and for suspensions, to provide acceptable viscosity to ensure adequate suspension of the particulate to enable uniform dosing.

Effervescent granules are typically formulated from sodium or potassium bicarbonate and an acid such as citric or tartaric acid. To prevent untimely generation of carbon dioxide, manufacturers should take special precautions to limit residual water in the product due to manufacture and to select packaging that protects the product from moisture. The manufacture of effervescent granules can require specialized facilities designed to maintain very low humidity (approximately 10% relative humidity). Effervescent powder mixtures are purposely formed into relatively course granules to reduce the rate of dissolution and provide a more controlled effervescence.

Reconstitution of granules must ensure complete wetting of all ingredients and sufficient time and agitation to allow the soluble components to dissolve. Specific instructions for reconstitution provided by the manufacturer should be carefully followed.

Reconstituted suspensions should be thoroughly mixed or shaken before use to resuspend the dispersed particulates. This is especially true of suspension preparations dosed from multiple-dose containers. For particularly viscous suspensions prone to air entrapment, instructions may advise the user how to shake the preparation to resuspend settled particulates while minimizing air entrapment.

For granules reconstituted to form suspensions for oral administration, acceptable suspension of the particulate phase depends on the particle size of the dispersed phase as well as the viscosity of the vehicle. Temperature can influence the viscosity, which influences suspension properties and the ease of removal of the dose from the bottles. In addition, temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. Thus, clear instructions should be provided regarding the appropriate storage temperature for the product.

Gums

Medicated gum is a pliable dosage form that is designed to be chewed rather than swallowed. Medicated gums release the drug substance(s) into the saliva. Medicated gums can deliver therapeutic agents for local action in the mouth or for systemic absorption via the buccal or gastrointestinal routes (e.g., nicotine or aspirin). Most gums are manufactured using the conventional melting process derived from the confectionary industry or alternatively may be directly compressed from gum powder. Medicated gums are formulated from insoluble synthetic gum bases such as polyisoprene, polyisobutylene, isobutyleneisoprene copolymer, styrene butadiene rubber, polyvinyl acetate, polyethylene, ester gums, or polyterpenes. Plasticizers and softeners such as propylene glycol, glycerin, oleic acid, or processed vegetable oils are added to keep the gum base pliable and to aid in the incorporation of the drug substance(s), sweeteners, and flavoring agents. Sugars as well as artificial sweeteners and flavorings are incorporated to improve taste, and dyes may be used to enhance appearance. Some medicated gums are coated with magnesium stearate to reduce tackiness and improve handling during packaging. A preservative may be added.

PREPARATION

Melted gum: The gum base is melted at a temperature of about 115° until it has the viscosity of thick syrup and, at that point, is filtered through a fine-mesh screen. This molten gum base is transferred to mixing tanks where the sweeteners, plasticizers, and typically the drug substance are added and mixed. Colorings, flavorings, and preservatives are added and mixed while the melted gum is cooling. The cooled mixture is shaped by extrusion or rolling and cutting. Dosage units of the desired shape and potency are packaged individually. Additional coatings such as powder coatings to reduce tackiness or film or sugar coatings may be added to improve taste or facilitate bulk packaging.

Directly compressed gum: The gum base is supplied in a free-flowing granular powder form. The powder gum base is then dry blended with sweeteners, flavors, the drug substance, and lubricant. The blend is then processed through a conventional tablet press and tableted into desired shapes. The resulting medicated gum tablets can be further coated with sugar or sugar-free excipients. These tablets can be packaged in blisters or bottles as needed.

SPECIAL CONSIDERATIONS

Medicated gums are typically dispensed in unit-dose packaging. The patient instructions also may include a caution to avoid excessive heat.

Implants

Implants are long-acting dosage forms that provide continuous release of the drug substance often for periods of months to years. They are administered by the parenteral route and are sterile. Some implants approved as animal drugs to be administered subcutaneously to the ears are not required to be sterile. Typically for systemic delivery, they may be placed subcutaneously, or for local delivery they can be placed in a specific region in the body (e.g., in the sinus, in an artery, in the eye, in the brain, etc.). Implants are usually administered by means of a suitable injector or by surgical procedure.

Polymer implants can be formed as a single-shaped mass such as a cylinder. The polymer matrix must be biocompatible (see *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩), but it can be either bioabsorbable or nonbioabsorbable. Shaped polymer implants are administered by means of a suitable special injector. Release kinetics are typically not zero-order, but zero-order kinetics are possible. Drug substance release can be controlled by the diffusion of the drug substance from the bulk polymer matrix or by the properties of a rate-limiting polymeric membrane coating. Polymer implants are used to deliver potent small molecules like steroids (e.g., estradiol for cattle) and large molecules like peptides [e.g., luteinizing hormone-releasing hormone (LHRH)]. Example durations of drug substance release are 2 and 3 months for bioabsorbable implants and up to 3 years for nonbioabsorbable implants. An advantage of bioabsorbable implants is that they do not require removal after the release of all drug substance content. Nonbioabsorbable polymer implants can be removed

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before or after a drug substance release is complete or may be left in situ. An implant can have a tab with a hole in it to facilitate suturing it in place (e.g., for an intravitreal implant for local ocular delivery). Such implants may provide therapeutic release for periods as long as 2.5 years.

Drug substance-eluting stents combine the mechanical effect of the stent to maintain arterial patency with the prolonged pharmacologic effect of the incorporated drug substance (to reduce restenosis, inhibit clot formation, or combat infection). As an example, a metal stent can be coated with a nonbioabsorbable or bioabsorbable polymer-containing drug substance. The resultant coating is a polymeric matrix that controls the extended release of the drug substance.

PREPARATION

Cylindrical polymeric implants are commonly made by melt extrusion of a blend of drug substance and polymer, resulting in a rod that is cut into shorter lengths. Polymer implants can also be made by injection molding. Still other implants are assembled from metal tubes and injection-molded plastic components.

Sterility can be achieved by terminal sterilization or by employing aseptic manufacturing procedures.

Injections

(See *Emulsions*, *Powders*, *Solutions*, and *Suspensions*.)

Injections are not treated as a dosage form in this chapter. Chapter ⟨1⟩ provides quality and other information about injectable products. Information on specific dosage form terminology can be found in the *Glossary*. For appropriate injection nomenclature, see *Nomenclature* ⟨1121⟩.

EXCESS VOLUME IN INJECTIONS

Each container of an injection is filled with a volume in slight excess of the labeled "size" or the volume that is to be withdrawn. The excess volumes recommended in *Table 1* are usually sufficient to permit withdrawal and administration of the labeled volumes.

Table 1

Labeled Size (mL)	Recommended Excess Volume	
	For Mobile Liquids (mL)	For Viscous Liquids (mL)
0.5	0.10	0.12
1.0	0.10	0.15
2.0	0.15	0.25
5.0	0.30	0.50
10.0	0.50	0.70
20.0	0.60	0.90
30.0	0.80	1.20
50.0 or more	2%	3%

Inserts

Inserts are solid dosage forms that are inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum (see *Suppositories*). The drug substance in inserts is delivered for local or systemic action. Vaginal inserts are usually globular or oviform and weigh about 5 g each. Inserts intended to dissolve in vaginal secretions are usually made from water-soluble or water-miscible vehicles such as polyethylene glycol or glycerinated gelatin.

PREPARATION

For general considerations, see ⟨795⟩. Inserts vary considerably in their preparation. Inserts may be molded (using technology similar to that used to prepare lozenges, suppositories, or plastics), compressed from powders (as in tableting), or formulated as special applications of capsules (soft gelatin capsules and hard gelatin capsules have been employed for extemporaneously compounded preparations). Inserts may be formulated to melt at body temperature or disintegrate upon insertion. Design of the dosage form should take into consideration the fluid volume available at the insertion site and minimize the potential to cause local irritation. Most inserts are formulated to ensure retention at the site of administration.

Irrigations

(See *Solutions*.)

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Liquids

As a dosage form, a liquid consists of a pure chemical in its liquid state. Examples include mineral oil, isoflurane, and ether. This dosage form term is not applied to solutions.

Lotions

(See *Emulsions*.)

Lozenges

Lozenges are solid oral dosage forms that are designed to dissolve or disintegrate slowly in the mouth. They contain one or more drug substances that are slowly liberated from the, typically, flavored and sweetened base. They are frequently intended to provide local action in the oral cavity or the throat but also include those intended for systemic absorption after dissolution. The typical therapeutic categories of drug substances delivered in lozenges are antiseptics, analgesics, decongestants, antitussives, and antibiotics. Molded lozenges are called cough drops or pastilles but these terms are not used in official article titles. Lozenges prepared by compression or by stamping or cutting from a uniform bed of paste are sometimes known as troches (a term not used in official article titles). Compressed or stamped lozenges are often produced in a circular shape.

Lozenges can be made using sugars such as sucrose and dextrose, or can provide the benefits of a sugar-free formulation that is usually based on sorbitol or mannitol. Polyethylene glycols and hypromellose are sometimes included to slow the rate of dissolution.

PREPARATION

Excipients used in molded lozenge manufacture include gelatin, fused sucrose, sorbitol, or another carbohydrate base.

Molded lozenges can be prepared by mixing the ingredients with water and heating to reduce the water content. The viscous solution is then poured into molds (e.g., corn starch molds). The lozenges are quickly cooled in the molds to trap the base in the glassy state. Once formed, the lozenges are removed from the molds and packaged. Care is taken to avoid excessive moisture during storage to prevent crystallization of the sugar base.

Compressed lozenges are made using excipients that may include a filler, binder, sweetening agent, flavoring agent, and lubricant. Sugars such as sucrose, sorbitol, and mannitol are often included because they can act as a filler and binder as well as serve as sweetening agents. Approved FD&C and D&C dyes or lakes (dyes adsorbed onto insoluble aluminum hydroxide) may also be present.

The manufacturing of compressed lozenges is essentially the same as that for conventional tableting, with the exception that a tablet press capable of making larger tablets and exerting greater force to produce harder tablets may be required (see *Tablets*).

The paste used to produce lozenges manufactured by stamping or cutting contains a moistening agent, sucrose, and flavoring and sweetening agents. The homogenous paste is spread as a bed of uniform thickness, and the lozenges are cut or stamped from the bed and are allowed to dry. Some lozenges are prepared by forcing dampened powders under low pressure into mold cavities and then ejecting them onto suitable trays for drying at moderate temperatures.

Ointments

Ointments are semisolid preparations generally intended for external application to the skin or mucous membranes. Drug substances delivered in ointments are intended for local action or for systemic absorption. Ointments usually contain less than 20% water and volatiles, and more than 50% hydrocarbons, waxes, or polyols as the vehicle. Ointment bases recognized for use as vehicles fall into four general classes: hydrocarbon bases, absorption bases, water-removable bases, and water-soluble bases.

HYDROCARBON BASES

Also known as oleaginous ointment bases, hydrocarbon bases allow the incorporation of only small amounts of an aqueous component. Ointments prepared from hydrocarbon bases act as occlusive dressings and provide prolonged contact of the drug substance with the skin. They are difficult to remove and do not change physical characteristics upon aging.

ABSORPTION BASES

Absorption bases allow the incorporation of aqueous solutions. Such bases include only anhydrous components (e.g., *Hydrophilic Petrolatum*) or W/O emulsions (e.g., *Lanolin*). Absorption bases are also useful as emollients.

WATER-REMOVABLE BASES

O/W emulsions (e.g., *Hydrophilic Ointment*) are sometimes referred to as creams (see *Emulsions*). Water-removable bases may be readily washed from the skin or clothing with water, making them acceptable for cosmetic reasons. Other advantages of the water-removable bases are that they can be diluted with water and that they favor the absorption of serous discharges in dermatological conditions.

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WATER-SOLUBLE BASES

Also known as greaseless ointment bases, they are formulated entirely from water-soluble constituents. *Polyethylene Glycol Ointment* is the only official preparation in this group. Water-soluble bases offer many of the advantages of the water-removable bases and, in addition, contain no water-insoluble substances such as petrolatum, anhydrous lanolin, or waxes. They are more correctly categorized as gels (see *Gels*).

The choice of an ointment base depends on the action desired, the characteristics of the incorporated drug substance, and the latter's bioavailability if systemic action is desired. The product's stability may require the use of a base that is less than ideal in meeting other quality attributes. Drug substances that hydrolyze rapidly, for example, are more stable in hydrocarbon bases than in bases that contain water.

PREPARATION

Ointments are typically prepared by either direct incorporation into a previously prepared ointment base or by fusion (heating during the preparation of the ointment). A levigating agent is often added to facilitate the incorporation of the medicament into the ointment base by the direct incorporation procedure. In the fusion method, the ingredients are heated. Homogenization is often necessary. The rate of cooling is an important manufacturing detail because rapid cooling can impart increased structure to the product of the fusion method.

Pastes

Pastes are semisolid preparations of stiff consistency and contain a high percentage (20%–50%) of finely dispersed solids. Pastes are intended for application to the skin, oral cavity, or mucous membranes. Pastes ordinarily do not flow at body temperature and thus can serve as occlusive, protective coatings. As a consequence, pastes are more often used for protective action than are ointments.

Fatty pastes that have a high proportion of hydrophilic solids appear less greasy and are more absorptive than ointments. They are used to absorb serous secretions and are often preferred for acute lesions that have a tendency toward crusting, vesiculation, or oozing.

Dental pastes are applied to the teeth. Other orally administered pastes may be indicated for adhesion to the mucous membrane for a local effect.

In veterinary medicine, pastes are typically administered orally and are intended for systemic delivery of drug substances. The paste is squeezed into the mouth of the animal, generally at the back of the tongue, or is spread inside the mouth.

Pellets

The use of the term "pellet" for implantable dosage forms is no longer preferred (see *Implants*). In veterinary medicine, medicated articles and feeds may be pelletized but are not considered dosage forms (see *Animal Drugs For Use In Animal Feeds* ⟨1152⟩).

Pellets are small solid dosage forms that can be designed as single or multiple entities. They can have a spherical or nearly spherical shape, although such a shape is not required. Spherical pellets are sometimes referred to as beads. Pellets used in veterinary medicine may instead be cylindrical in shape. Pellets can provide several advantages, including physical separation for chemically or physically incompatible materials and for control of the release of drug substance. Pellets may be designed with the drug substance dispersed in a matrix or the pellets may be coated with an appropriate polymer. Pellets may be administered by the oral (gastrointestinal) route. Pellets for oral administration can:

1. Protect stomach tissues from irritation
2. Sometimes minimize variability associated with gastric retention of larger dosage forms
3. Solely extend the release of the drug substance
4. Solely delay the release
5. Both extend and delay the release of the drug substance

Some pellets can be sprinkled on food. In the case of delayed-release formulations, the coating polymer is chosen to resist dissolution at the lower pH of the gastric environment but to dissolve in the higher pH of the intestinal environment.

Pellets may be administered by injection. One or several pellets can be injected or surgically administered to provide continuous therapy for periods of months or years (see *Implants*).

In veterinary medicine, pellets may be used to improve palatability of the drug product and pellets for oral administration may be delivered on top of an individual animal's food or feed.

PREPARATION

The desired performance characteristics determine the manufacturing method chosen. In general, pellet dosage forms are manufactured by compression, or by wet or dry extrusion processes sometimes followed by spheronization, or followed by wet or dry coating processes. Manufacture of pellets by wet coating usually involves the application of successive coatings upon nonpareil seeds. This manufacturing process is frequently conducted in fluid-bed processing equipment. Dry powder coating or layering processes are often performed in specialized rotor granulation equipment. The extent of particle growth achievable in wet coating processes is generally more limited than the growth that can be obtained with dry powder layering techniques, but either method allows the formulator to develop and apply multiple layers of coatings to achieve the desired release profile. The manufacture of pellets by compression is largely restricted to the production of material for subcutaneous implantation. This method of manufacture provides the necessary control to ensure dose uniformity and is generally better suited to aseptic processing requirements.

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Alternatively, microencapsulation techniques can be used to manufacture pellets. Coacervation coating techniques typically produce coated particles that are much smaller than those made by other techniques.

Pills

Pills are drug substance-containing small, spherical, solid bodies intended for oral administration. The pill dosage form has been largely replaced by compressed tablets and by capsules. Unlike tablets, pills are usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms, such as tablets and capsules.

PREPARATION

Excipients are selected on the basis of their ability to produce a mass that is firm and plastic. The drug substance is triturated with powdered excipients in serial dilutions to attain a uniform mixture. Liquid excipients that act to bind and provide plasticity to the mass are subsequently added to the dry materials. The mass is formed by kneading. The properties of firmness and plasticity are necessary to permit the mass to be worked and retain the shape produced. Cylindrical pill pipes are produced from portions of the mass. The pill pipe is cut into individual lengths corresponding to the intended pill size, and the pills are rolled to form the final shape. Pill-making machines can automate the preparation of the mass, production of pill piping, and the cutting and rolling of pills.

Plasters

A plaster is a semisolid substance for external application that is supplied on a support material. Plasters are applied for prolonged periods to provide protection, support, or occlusion (maceration). This term is not preferred and should not be used for new drug product titles. Plasters consist of an adhesive layer that may contain active substances. This layer is spread uniformly on an appropriate support that is usually made of a rubber base or synthetic resin. Unmedicated plasters are designed to provide protection or mechanical support to the site of application. Plasters are available in a range of sizes or cut to size to effectively provide prolonged contact to the site of application. They adhere firmly to the skin but can be peeled off the skin without causing injury.

Powders

Powders are defined as a single solid or a mixture of solids in a finely divided state. Powders used as pharmaceutical dosage forms may contain one or more drug substances and can be used as is or can be mixed with a suitable vehicle for administration. (See *Solutions* or *Suspensions*.) Powders can be intended for internal or external use. Powders for external use are typically dusted onto the skin or applied to bandages or clothing. Powders for internal use can be applied to accessible mucous membranes with suitable applicators or are entrained in air streams for application to the nose or lungs.

The performance of powder dosage forms can be affected by the physical characteristics of the powder. Selection of relevant and appropriate powder characteristics depends on the dosage form and its route of administration. For example, particle size can influence the dissolution rate of the particles and thus the bioavailability and/or effectiveness at the site of action. Externally applied powders should have a particle size of 150 µm or less (typically in the 50- to 100-µm range to prevent a gritty feel on the skin that could further irritate traumatized skin). The particle size of powders delivered to the lung or nose influences where the powder is deposited. Particle size may influence the mixing, segregation, and aggregation of the particles, which can affect the delivery and uniformity of the dosage form. For more information, see *Powder Fineness* ⟨811⟩ and ⟨5⟩.

In veterinary medicine, a powder that needs to be reconstituted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term "concentrate" is no longer preferred.

INHALATION POWDERS AND NASAL POWDERS

Inhalation powders and nasal powders consist of an appropriately finely divided solid and a suitable container–closure delivery system. For additional information, see ⟨5⟩ and ⟨601⟩.

PREPARATION

Powder dosage forms can be produced by the combination of multiple components into a uniform blend. This preparation can also involve particle size reduction, a process referred to as comminution. Milling, spray drying, supercritical fluid, high-pressure homogenization, precipitation technologies, and porous microparticle fabrication techniques may be used to reduce the particle size of powders. As the particle size is decreased, the number of particles and the surface area increase, which can increase the dissolution rate and bioavailability, and/or the rate and extent of local action, of the drug substance.

Blending of powders may be accomplished by different techniques. Industrial processes may employ sifting or tumbling the powders in a rotating container. One of the most common tumble blenders is a V-blender, which is available in a variety of sizes suitable for small-scale and large-scale compounding and industrial production. Depending on the particle size of the drug substance, a random mixture of powders may be employed. Blending techniques for powders include those used in compounding pharmacy such as spatulation and trituration (see ⟨795⟩).

Powder flow can be influenced by both particle size and shape. Larger particles generally flow more freely than do fine particles. Powder flow is an important attribute that can affect the packaging or dispensing of a powder.

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Rinses

(See *Solutions*.)

Soaps and Shampoos

Soaps and shampoos are solid or liquid preparations intended for topical application to the skin or scalp followed by subsequent rinsing with water. Soaps and shampoos are emulsions, suspensions, or surface-active compositions that readily form emulsions, micelles, or foams upon the addition of water followed by rubbing. Incorporation of drug substances in soaps and shampoos combines the cleansing/degreasing abilities of the vehicle and facilitates the topical application of the drug substance to affected areas, even large areas, of the body. The surface-active properties of the vehicle facilitate contact of the drug substance with the skin or scalp. Medicated soap and shampoo formulations frequently contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination.

PREPARATION

The preparation of medicated soaps and shampoos follows techniques frequently used for the preparation of emulsified systems. To ensure uniformity, the drug substance(s) must be added to the vehicle prior to congealing (in the case of soaps) followed by thorough mixing. If the medication is present as a suspension, the particle size must be controlled to promote uniform distribution of the drug substance and possibly optimize performance. Because soap manufacture frequently involves processing the ingredients at an elevated temperature, care must be exercised to avoid excessive degradation of the drug substance during processing.

Solutions

A solution is a preparation that contains one or more dissolved chemical substances in a suitable solvent or mixture of mutually miscible solvents. Because molecules of a drug substance in solution are uniformly dispersed, the use of solutions as dosage forms generally provides assurance of uniform dosage upon administration and good accuracy when the solution is diluted or otherwise mixed.

Substances in solutions are more susceptible to chemical instability than they are in the solid state and, dose-for-dose, are generally heavier and more bulky than solid dosage forms. These factors increase the cost of packaging and shipping relative to that of solid dosage forms. Some solutions are prepared and ready for use, and others are prepared as powders or other solids intended for reconstitution with an appropriate vehicle just before use (see *Powders*). Solution dosage forms can be administered by injection, inhalation, and the mucosal, topical, and gastrointestinal routes. A solution administered by injection is officially titled "injection" (see ⟨1⟩).

Some solutions are designed to form a mass in situ. These solutions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the solution to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Solutions intended for oral administration usually contain flavorings and colorants to make the medication more attractive and palatable for the patient or consumer. When needed, they also may contain stabilizers to maintain chemical and physical stability and preservatives to prevent microbial growth.

Solutions are sometimes placed on devices such as swabs, cloths, or sponges, that aid application.

In veterinary medicine, a solution that needs to be diluted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term "concentrate" is no longer preferred.

Sprays

Spray preparations may deliver either accurately metered or nonmetered amounts of formulation.

A spray drug product is a dosage form that contains a drug substance in the liquid state as a solution or suspension and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

Depending on the design of the formulation and the valve system, the droplets generated may be intended for immediate inhalation through the mouth and deposition in the pulmonary tree, or for inhalation into the nose and deposition in the nasal cavity.

The mechanism for droplet generation and the intended use of the preparation distinguish various classes of sprays. A spray may be composed of a pump, container, actuator, valve, nozzle, or mouthpiece in addition to the formulation containing the drug(s), solvent(s), and any excipient(s). The design of each component plays a role for the appropriate performance of the drug product and in determining the critical characteristics of the droplet size distribution. Droplet and particle size distributions, delivered dose uniformity, plume geometry, and droplet velocity are critical parameters that influence the efficiency of drug delivery. When the preparation is supplied as a multidose container, the addition of a suitable antimicrobial preservative may be necessary. Spray formulations intended for local or systemic effect typically have an aqueous base and may contain excipients to control pH and viscosity. In addition, depending on the route of administration, the formulation may be isotonic. For additional information, see ⟨5⟩ and ⟨601⟩.

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LABELING AND USE

Refer to the Center for Drug Evaluation and Research (CDER) *Guidance for Industry: Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products—Chemistry, Manufacturing, and Controls Documentation.*

Strips

A strip is a dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper. Typically it is sterile and it may be impregnated with a compound or be gauged to allow measurements for diagnostic purposes, such as in measuring tear production. The term "strip" should not be used when another term such as "film" is more appropriate.

Suppositories

Suppositories are dosage forms adapted for application into the rectum. They melt, soften, or dissolve at body temperature. A suppository may have a local protectant or palliative effect, or may deliver a drug substance for systemic or local action.

Suppository bases typically include cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights, and fatty acid esters of polyethylene glycol. The suppository base can have a notable influence on the release of the drug substance(s). Although cocoa butter melts quickly at body temperature, it is immiscible with body fluids and this inhibits the diffusion of fat-soluble drug substances to the affected sites. Polyethylene glycol is a suitable base for some antiseptics. In cases when systemic action is desired, incorporating the ionized rather than the nonionized form of the drug substance may help maximize bioavailability. Although nonionized drug substances partition more readily out of water-miscible bases such as glycerinated gelatin and polyethylene glycol, the bases themselves tend to dissolve very slowly, which slows drug substance release. Cocoa butter and its substitutes (e.g., *Hard Fat*) perform better than other bases for allaying irritation in preparations intended for treating internal hemorrhoids. Suppositories for adults are tapered at one or both ends and usually weigh about 2 g each.

PREPARATION

Cocoa butter suppositories have cocoa butter as the base and can be made by incorporating the finely divided drug substance into the solid oil at room temperature and suitably shaping the resulting mass, or by working with the oil in the melted state and allowing the resulting suspension to cool in molds. A suitable quantity of hardening agents may be added to counteract the tendency of some drug substances (such as chloral hydrate and phenol) to soften the base. The finished suppository melts at body temperature.

A variety of vegetable oils, such as coconut or palm kernel, modified by esterification, hydrogenation, or fractionation, are used as cocoa butter substitutes to obtain products that display varying compositions and melting temperatures (e.g., *Hydrogenated Vegetable Oil* and *Hard Fat*). These products can be designed to reduce rancidity while incorporating desired characteristics such as narrow intervals between melting and solidification temperatures, and melting ranges to accommodate formulation and climatic conditions.

Drug substances can be incorporated into glycerinated gelatin bases by addition of the prescribed quantities to a vehicle consisting of about 70 parts of glycerin, 20 parts of gelatin, and 10 parts of water.

Several combinations of polyethylene glycols that have melting temperatures that are above body temperature are used as suppository bases. Because release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in preparation and storage than is the case for melting-type vehicles. However, high concentrations of higher molecular weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention.

Several nonionic surface-active agents closely related chemically to the polyethylene glycols can be used as suppository vehicles. Examples include polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene stearates. These surfactants are used alone or in combination with other suppository vehicles to yield a wide range of melting temperatures and consistencies. A notable advantage of such vehicles is their water dispersibility. However, care must be taken with the use of surfactants because they may either increase the rate of drug substance absorption or interact with the drug substance to reduce therapeutic activity.

Compounding suppositories using a suppository base typically involves melting the suppository base and dissolution or dispersion of the drug substance in the molten base (see ⟨795⟩). When compounding suppositories, the compounding professional prepares an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. In compounding suppositories, avoid caustic or irritating ingredients, carefully select a base that will allow the drug substance to provide the intended effect, and in order to minimize abrasion of the rectal membranes, reduce solid ingredients to the smallest reasonable particle size.

Suspensions

A suspension is a biphasic preparation consisting of solid particles dispersed throughout a liquid phase. Suspension dosage forms may be formulated for specific routes of administration such as oral, topical, inhalation, ophthalmic, otic, and injection. Some suspensions are prepared and ready for use, and others are prepared as powders or other solids intended for reconstitution with an appropriate vehicle just before use (see *Powders*).

Inhalation suspensions (see ⟨5⟩), ophthalmic suspensions, injectable suspensions, and some otic suspensions are prepared in sterile form. Suspensions are generally not injected intravenously, epidurally, or intrathecally unless the product labeling clearly specifies these routes of administration.

Some liposomal drug products are referred to as suspensions because they can settle and require resuspension prior to administration (see ⟨1⟩).

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Resorbable microparticles can provide extended release of a drug substance over periods varying from a few weeks to months. They can be administered subcutaneously or intramuscularly for systemic delivery, or they may be deposited in a desired location in the body for site-specific delivery. Resorbable microparticles (or microspheres) generally range from 20 to 100 µm in diameter. They are composed of a drug substance dispersed within a biocompatible, bioabsorbable polymeric excipient (matrix). Poly(lactide-co-glycolide) polymers have been used frequently. These excipients typically resorb by hydrolysis of ester linkages. The microparticles are typically administered by suspension in an aqueous vehicle followed by injection with a conventional syringe and needle. Release of the drug substance from the microparticles begins after physiological fluid enters the polymer matrix, dissolving some of the drug substance that is then released by a diffusion-controlled process. Drug release also can occur as the bioresorbable polymer molecular weight decreases and as the matrix erodes.

Some suspensions are designed to form a mass in situ. These suspensions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the suspension to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Historically, the term "milk" was sometimes used for suspensions in aqueous vehicles intended for oral administration (e.g., *Milk of Magnesia*). The term "magma" is often used to describe suspensions of inorganic solids, such as clays in water, that display a tendency toward strong hydration and aggregation of the solid, giving rise to gel-like consistency and thixotropic rheological behavior (e.g., *Bentonite Magma*). In the past, the term "lotion" referred to both topical suspensions and topical emulsions. Now the term only refers to topical emulsions (see *Emulsions*).

Limited aqueous solubility of the drug substance(s) is the most common rationale for developing a suspension. Other potential advantages of an oral suspension include taste masking and improved patient compliance because of the more convenient dosage form. When compared to solutions, suspensions can have improved chemical stability. Ideally, a suspension should contain small uniform particles that are readily suspended and easily redispersed following settling. Unless the dispersed solid is colloidal, the particulate matter in a suspension will likely settle to the bottom of the container upon standing. Such sedimentation may lead to caking and solidification of the sediment and difficulty in redispersing the suspension upon agitation. To prevent such problems, manufacturers commonly add ingredients to increase viscosity and the gel state of the suspension or flocculation, including clays, surfactants, polyols, polymers, or sugars. Frequently, thixotropic vehicles are used to counter particle-settling tendencies, but these vehicles must not interfere with pouring or redispersal. Additionally, the density of the dispersed phase and continuous phase may be modified to further control settling rate. For topical suspensions, rapid drying upon application is desirable.

Temperature can influence the viscosity (and thus suspension properties and the ease of removing the dose from the bottle), and temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. When manufacturers conduct stability studies to establish product shelf life and storage conditions, they should cycle conditions (freeze/thaw) to investigate temperature effects.

Unless studies confirm that the formulation will not support microbial growth, suspension preparations packaged to provide multiple doses should contain suitable antimicrobial agents to protect against bacterial, yeast, and mold contamination (see ⟨51⟩) or other appropriate measures should be taken to avoid microbial contamination.

Suspensions for reconstitution are dry powder or granular mixtures that require the addition of water or a supplied formulated diluent before administration. This formulation approach is frequently used when the chemical or physical stability of the drug substance or suspension does not allow sufficient shelf life for a preformulated suspension. Typically, these suspensions are refrigerated after reconstitution to increase their shelf life. For this type of suspension, the powder blend is uniform and the powder readily disperses when reconstituted.

Injectable suspensions are generally intended for either subcutaneous or intramuscular routes of administration and should have a controlled particle size, typically in the range of 5 µm or smaller. The rationale for the development of injectable suspensions may include poor drug substance solubility, improved chemical stability, prolonged duration of action, and avoidance of first-pass metabolism. Care is needed in selecting the sterilization technique because it may affect product stability or alter the physical properties of the material.

In veterinary medicine, a suspension that needs to be diluted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term "concentrate" is no longer preferred.

PREPARATION

Suspensions are prepared by adding suspending agents or other excipients and purified water or oil to solid drug substances and mixing to achieve uniformity. In the preparation of a suspension, the characteristics of both the dispersed phase and the dispersion medium should be considered. During development, manufacturers should define an appropriate particle size distribution for the suspended material to achieve the desired effectiveness and to minimize the likelihood of particle size changes during storage.

In some instances, the dispersed phase has an affinity for the vehicle and is readily wetted upon its addition. For some materials, the displacement of air from the solid surface is difficult, and the solid particles may clump together or float on top of the vehicle. In the latter case, a wetting agent may be used for certain types of suspensions to facilitate displacement of air from the powder surface. Surfactants, alcohol, glycerin, and other hydrophilic liquids can be used as wetting agents when an aqueous vehicle will be used as the dispersion phase. These agents function by displacing the air in the crevices of the particles and dispersing the particles. In the large-scale preparation of suspensions, wetting of the dispersed phase may be aided by the use of high-energy mixing equipment such as colloid mills or other rotor–stator mixing devices.

After the powder has been wetted, the dispersion medium (containing the soluble formulation components such as colorants, flavorings, and preservatives) is added in portions to the powder, and the mixture is thoroughly blended before subsequent additions of the vehicle. A portion of the vehicle is used to wash the mixing equipment free of suspended material, and this portion is used to bring the suspension to final volume and ensure that the suspension contains the desired concentration of solid matter. The final product may be passed through a colloid mill or other blender or mixing device to ensure uniformity.

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Suspensions are resuspended before the dose is dispensed. Because of the viscosity of many suspension vehicles, air entrainment may occur during dosing. The formulation process allows evaluation of this possibility; adjustments in vehicle viscosity or the incorporation of low levels of antifoaming agents are common approaches to minimize air entrainment. Alternatively, specific instructions for resuspending the formulation may be provided to minimize air incorporation and ensure accurate dosing.

Systems

Systems are preparations of drug substance(s) in carrier devices, often containing adhesive backing, that are applied topically or inserted into body cavities. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use. The term "system" should not be used when another dosage form term is more appropriate (e.g., inserts and implants).

The notation of strength is either defined in terms of the amount of the drug substance released from the system over a specific period of time or as the drug concentration within the formulation (e.g., the percentage of the drug). Various routes of administration are possible, so the route must always be indicated in the compendial name when a specific location for application is essential for proper use (e.g., "intrauterine", "ocular", or "periodontal" as the route of administration). For example, systems applied to the eye are called ocular systems. The route is named "transdermal" when, for example, systemic absorption of the drug substance may take place through the dermis without specifying the region of the body to which the system is applied.

The term "patch" has sometimes been used but is not preferred for use in drug product monograph nomenclature when referring to a system.

Intrauterine systems are intended for placement in the uterus. Release of the drug substance can be up to 5 years.

Ocular systems are intended for placement in the lower conjunctival fornix from which the drug diffuses through a membrane at a constant rate.

Periodontal systems are intended for placement in the pocket between the tooth and the gum. In some cases, periodontal systems may be formed in situ in the periodontal pocket and release the drug substance(s) for several weeks.

Transdermal systems (TDS) are placed onto intact skin to deliver the drug to the systemic circulation. They are designed for prolonged release (up to 7 days). Specific quality tests for TDS are found in ⟨3⟩.

Tablets

Tablets are solid dosage forms in which the drug substance is generally blended with excipients and compressed into the final dosage. Tablets are the most widely used dosage form in the United States. Tablet presses use steel punches and dies to prepare compacted tablets by the application of high pressures to powder blends or granulations. Tablets can be produced in a wide variety of sizes, shapes, and surface markings. Capsule-shaped tablets are commonly referred to as caplets, although the term is not used in official article titles. Specialized tablet presses may be used to produce tablets with multiple layers or with specially formulated core tablets placed in the interior of the final dosage form. These specialized tablet presentations can delay or extend the release of the drug substance(s) or physically separate incompatible drug substances. Tablets may be coated by a variety of techniques to provide taste masking, protection of photo-labile drug substance(s), extended or delayed release, or unique appearance (colors). When no deliberate effort has been made to modify the drug substance release rate, tablets are referred to as immediate-release.

BUCCAL TABLETS

Intended to be inserted in the buccal pouch, where the drug substance is absorbed directly through the oral mucosa. Few drug substances are readily absorbed in this way (examples are nitroglycerin and certain steroid hormones).

CHEWABLE TABLETS

Formulated and manufactured to produce a pleasant-tasting residue in the mouth and to facilitate swallowing. Hard chewable tablets are typically prepared by compaction, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and contain colors and flavors to enhance their appearance and taste. Soft chewable tablets are typically made by a molding or extrusion process, frequently with more than 10% water to help maintain a pliable, soft product. Hard chewable tablets in veterinary medicine often have flavor enhancers like brewer's yeast or meat/fish-based flavors.

Tablets for human use that include "chewable" in the title must be chewed or crushed prior to swallowing to ensure reliable release of the drug substance(s) or to facilitate swallowing. If tablets are designed so that they may be chewed (but chewing is not required for drug substance release or ease of swallowing), the title should not include a reference to "chewable". In that case, the product may still be described as "chewable" in the ancillary labeling statement.

Tablets for veterinary use that are intended to be chewed will include "Chewable" in the title. However, it is understood that for veterinary products it is not possible to ensure that tablets are chewed prior to ingestion. Chewable tablets may be broken into pieces and fed to animals that normally swallow treats whole.

EFFERVESCENT TABLETS

Prepared by compaction and contain, in addition to the drug substance(s), mixtures of acids (e.g., citric acid or tartaric acid) and carbonates, and/or sodium bicarbonate. Upon contact with water, these formulations release carbon dioxide, producing the characteristic effervescent action.

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HYPODERMIC TABLETS

Molded tablets made from completely and readily water-soluble ingredients; formerly intended for use in making preparations for hypodermic injection. They may be administered orally or sublingually when rapid drug substance availability is required.

MODIFIED-RELEASE TABLETS

There are two categories of modified-release tablet formulations recognized by USP.
Delayed-release tablets: Tablets are sometimes formulated with acid-resistant or enteric (also called "gastro-resistant") coatings to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation.
Extended-release tablets: Extended-release tablets are formulated in such a manner as to make the drug substance available over an extended period of time following ingestion. Requirements for dissolution (see ⟨711⟩) are typically specified in the individual monographs.

ORALLY DISINTEGRATING TABLETS

Orally disintegrating tablets are intended to disintegrate rapidly within the mouth to provide a dispersion before the patient swallows the resulting slurry where the drug substance is intended for gastrointestinal delivery and/or absorption. Some of these dosage forms have been formulated to facilitate rapid disintegration and are manufactured by conventional means or by using lyophilization or molding processes. Further details may be found in the CDER *Guidance for Industry: Orally Disintegrating Tablets.*

SUBLINGUAL TABLETS

Sublingual tablets are intended to be inserted beneath the tongue, where the drug substance is absorbed directly through the oral mucosa. As with buccal tablets, few drug substances are extensively absorbed in this way, and much of the drug substance is swallowed and is available for gastrointestinal absorption.

TABLETS FOR ORAL SOLUTION

Before administration, tablets for oral solution are intended to be solubilized in a liquid diluent. In some cases, tablets for oral solution may also be chewed or swallowed.

TABLETS FOR ORAL SUSPENSION

Tablets for oral suspension are intended to be dispersed in a liquid before administration as a suspension. The dosage form is tablets for oral suspension when either the drug substance or the excipients do not dissolve when dispersed in a liquid. In some cases, tablets for oral suspension may also be chewed or swallowed.

TABLET TRITURATES

Small, usually cylindrical, molded or compacted tablets. Tablet triturates traditionally were used as dispensing tablets in order to provide a convenient, measured quantity of a potent drug substance for compounding purposes, but they are rarely used today.

PREPARATION

Most compacted (compressed) tablets consist of the drug substance(s) and a number of excipients. These excipients may include fillers (diluents), binders, disintegrating agents, lubricants, and glidants. Approved FD&C and D&C dyes or lakes, flavors, and sweetening agents may also be present.

Fillers or diluents are added when the quantity of drug substance(s) is too small or the properties of the drug substance do not allow satisfactory compaction in the absence of other ingredients. Binders impart adhesiveness to the powder blend and promote tablet formation and maintenance of drug substance uniformity in the tableting mixture. Disintegrating agents facilitate reduction of the tablet into small particles upon contact with water or biological fluids. Lubricants reduce friction during the compaction and ejection cycles. Glidants improve powder fluidity, powder handling properties, and tablet weight control. Colorants are often added to tablet formulations for aesthetic value or for product identification.

Tablets are prepared from formulations that have been processed by one of three general methods: wet granulation, dry granulation (roll compaction or slugging), and direct compression.

Wet granulation: Involves the mixing of dry powders with a granulating liquid to form a moist granular mass that is dried and sized prior to compression. It is particularly useful in achieving uniform blends of low-dose drug substances and facilitating the wetting and dissolution of poorly soluble, hydrophobic drug substances.

Dry granulation: Can be produced by passing powders between rollers at elevated pressure (roll compaction). Alternatively, dry granulation can also be carried out by the compaction of powders at high pressures on tablet presses, a process also known as slugging. In either case, the compacts are sized before compression. Dry granulation improves the flow and handling properties of the powder formulation without involving moisture in the processing.

Direct compression: Tablet processing involves dry blending of the drug substance(s) and excipients followed by compression. The simplest manufacturing technique, direct compression, is acceptable only when the drug substance and excipients possess acceptable flow and compression properties without prior process steps.

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Tablets may be coated to protect the ingredients from air, moisture, or light; to mask unpleasant tastes and odors; to improve tablet appearance; and to reduce dustiness. In addition, coating may be used to protect the drug substance from acidic pH values associated with gastric fluids or to control the rate of drug release in the gastrointestinal tract.

The most common coating in use today is a thin film coating composed of a polymer that is derived from cellulose. Sugar coating is an alternative, less common approach. Sugar-coated tablets have considerably thicker coatings that are primarily sucrose with a number of inorganic diluents. A variety of film-coating polymers are available and enable the development of specialized release profiles. These formulations are used to protect acid-labile drug substances from the acidic stomach environment as well as to prolong the release of the drug substance to reduce dosing frequency (see ⟨711⟩ or ⟨701⟩).

Tapes

A tape is a dosage form suitable for delivering drug substances to the skin. It consists of a drug substance(s) impregnated into a durable yet flexible woven fabric or extruded synthetic material that is coated with an adhesive agent. Typically the impregnated drug substance is present in the dry state. The adhesive layer is designed to hold the tape securely in place without the aid of additional bandaging. Unlike transdermal systems, tapes are not designed to control the release rate of the drug substance. The term "tape" is not preferred and should not be used for new official article titles.

The drug substance content of tapes is expressed as amount per surface area with respect to the tape surface exposed to the skin. The use of an occlusive dressing with the tape enhances the rate and extent of delivery of the drug substance to deeper layers of the skin and may result in greater systemic absorption of the drug substance.

GLOSSARY

This glossary provides definitions for terms in use in medicine and serves as a source of official titles for official articles, except when the definition specifically states that the term is not to be used in drug product titles. Examples of general nomenclature forms for the more frequently encountered categories of dosage forms appear in ⟨1121⟩. In an attempt to be comprehensive, this glossary was compiled without the limits imposed by current preferred nomenclature conventions. To clearly identify/distinguish preferred from not preferred terms, entries indicate when a term is not preferred and generally direct the user to the current preferred term. Descriptive terms are used to identify a specialized presentation or characteristic of a dosage form. For example, the descriptive term "chewable" may be used with the dosage form "tablets" to identify a specific type of tablet that must be chewed prior to swallowing.

Aerosol: A dosage form consisting of a liquid or solid preparation packaged under pressure and intended for administration as a fine mist. When not used in naming, the term "aerosol" also refers to the fine mist of small droplets or solid particles that are emitted from the product.

Aromatic water (see *Solution*)**:** A clear, saturated, aqueous solution of volatile oils or other aromatic or volatile substances. The term is not used in official article titles.

Aural (Auricular) (see *Otic*)**:** For administration into, or by way of, the ear. The term is not used in official article titles.

Bead (see *Pellet*)**:** A solid dosage form in the shape of a small sphere. In most products a unit dose consists of multiple beads. The term is not used in official article titles.

Bolus (not preferred; see *Tablet*)**:** A large tablet intended for administration to large animals. Occasionally, the term "bolus" is used to describe a method of administration.

Buccal: Administration directed toward the cheek, generally from within the mouth.

Caplet (see *Tablet*)**:** Tablet dosage form in the shape of a capsule. The term is not used in official article titles.

Capsule: A solid dosage form in which the drug substance, with or without other ingredients, is filled into either a hard or soft shell or coated on the capsule shell. Most capsule shells are composed mainly of gelatin.

Chewable: A term for a solid dosage form that is intended to be chewed or crushed before swallowing.

Chewable gel: Formed or molded oral gel dosage forms that maintain their shape, are elastic, and yield to mastication. Chewable gels are also known as "gummies" but that term is not used for official article titles.

Coating (Coated): A term for the outer solid covering applied to a solid dosage form. This outer deposit is also referred to as a film. Coatings are applied for functional or aesthetic purposes such as taste masking, stability, modifying release characteristics, product identification, and appearance. The term is not used in official article titles.

Collodion (not preferred; see *Solution*)**:** A preparation that is a solution dosage form composed of pyroxilin dissolved in a solvent mixture of alcohol and ether, and applied externally.

Colloidal dispersion: A term for a preparation or formulation in which particles of colloidal dimension (i.e., typically between 1 nm and 1 µm) are distributed uniformly throughout a liquid.

Concentrate (not a preferred term for human or veterinary drug products)**:** The current use is for drug substances that are not intended for direct administration to humans or animals. The use in drug product nomenclature is being phased out (see ⟨1121⟩ and *Nomenclature Guidelines*[1]).

Conventional-release (see *Immediate-release*)**:** A term describing a dosage form in which no deliberate effort has been made to modify the release rate of the drug substance. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification. The term is not used in official article titles.

Cough drop (see *Lozenge*)**:** The term is not used in official article titles.

Cream: A semisolid emulsion dosage form often containing more than 20% water and volatiles, and/or containing less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or mucous membranes.

Delayed-release: A type of modified-release dosage form. When used in naming dosage forms, this term denotes a dosage form deliberately formulated to delay release of the drug substance for some period of time after initial administration. For oral products, expressions such as "enteric-coated" or "gastro-resistant" have been used where release of the drug substance is prevented in the gastric environment but promoted in the intestinal environment. However, the term "delayed-release" is used for official article titles.

Dental: When used in naming dosage forms, this term denotes a preparation that is applied to the teeth for localized action.

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Dip (not preferred; see *Immersion*)

Dispersible tablet (see *Tablet*, *Tablet for oral suspension*, or *Tablet for oral solution*): The term is not used in official article titles.

Disintegrating tablet (see *Tablet*, *Tablet for oral suspension*, or *Tablet for oral solution*; see also *Orally disintegrating*): The term is not used in official article titles.

Dosage form: A combination of drug substance(s) and/or excipient(s) in quantities and physical form designed to allow the accurate and efficient administration of the drug substance to the human or animal patient. The term is not used in official article titles.

Dry powder inhaler: A device used to administer an inhalation powder in a finely divided state suitable for oral inhalation by the patient. This term is not used in official article titles.

Effervescent: A term for an oral dosage form, frequently tablets or granules, containing ingredients that, when in contact with water, rapidly release carbon dioxide. The dosage form is dissolved or dispersed in water to initiate the effervescence prior to ingestion.

Elixir (not preferred; see *Solution*): A preparation that typically is a clear, flavored, sweetened hydroalcoholic solution intended for oral use. The term should not be used for new drug products in *USP–NF* but is commonly encountered in compounding pharmacy practice.

Emollient: A term for a cream or ointment indicating an increase in the moisture content of the skin following application of bland, fatty, or oleaginous substances. This term should not be used in official article titles.

Emulsion: A dosage form consisting of a two-phase system composed of at least two immiscible liquids, one of which is dispersed as droplets (internal or dispersed phase) within the other liquid (external or continuous phase), generally stabilized with one or more emulsifying agents. Emulsion is not used as a dosage form term if a more specific term is applicable (e.g., *Cream*, *Lotion*, or *Ointment*).

Enteric-coated (see *Delayed-release*): A term for a solid dosage form in which a polymer coating has been applied to prevent the release of the drug substance in the gastric environment. This term is not used in official article titles.

Excipient: An ingredient of a dosage form other than a drug substance. This term is not used in official article titles. The term "excipient" is synonymous with inactive ingredient.

Extended-release: A term denoting a dosage form that is deliberately formulated to prolong the release of the drug substance compared to that observed for an immediate-release dosage form. Expressions such as "prolonged release", "repeat action", "controlled release", "long acting", and "sustained release" have also been used to describe such dosage forms. However, the term "extended-release" is used for official article titles.

Extended-release injectable suspension: Liquid preparations of solids suspended in a suitable vehicle and formulated to allow the drug substance to be available over an extended period of time. The term "for extended-release injectable suspension" indicates dry solids that, upon the addition of a suitable vehicle, yield a preparation that conforms in all respects to the requirements for extended-release injectable suspensions.

Film: A term used to describe a thin sheet of material, usually composed of a polymer. Films are used in various routes of administration including as a means of oral administration of material in a rapidly dissolving form.

Foam: A dosage form containing gas dispersed in a liquid or solid continuous phase. Foams are formed at the time of application by dispensing product from the canister or other appropriate container and can be formulated to quickly break down into a liquid or to remain as a foam to ensure prolonged contact.

Gas: One of the states of matter having no definite shape or volume and occupying the entire container when confined. The term is not used in official article titles.

Gastro-resistant (see *Delayed-release*): A term for a solid dosage form in which a polymer coating has been applied to prevent release in the gastric environment. The term is not used in official article titles.

Gel: A dosage form that is a semisolid dispersion of small particles or a solution of large molecules interpenetrated by a solution containing a gelling agent to provide stiffness.

Gelcap: A capsule that is coated is sometimes referred to as a gelcap. Gelcap is not a term used in official article titles.

Geltab/Filmtab: A tablet that is coated is sometimes referred to as a geltab or filmtab. Geltab and filmtab are not terms used in official article titles.

Granules: A dosage form composed of dry aggregates of powder particles that may contain one or more drug substances, with or without other ingredients. They may be swallowed as such, dispersed in food, or dissolved in water. Granules are frequently compacted into tablets or filled into capsules, with or without additional ingredients. More commonly, granules are reconstituted as suspensions.

Gum: A dosage form in which the base consists of a pliable material that, when chewed, releases the drug substance into the oral cavity.

Gummies (see *Chewable gel*): The term is not used in official article titles.

Hard-shell capsule (not preferred; see *Capsule*): A type of capsule in which one or more drug substances, with or without other ingredients, are filled into a two-piece shell. Most hard-shell capsules are composed mainly of gelatin and are fabricated prior to the filling operation.

Immediate-release: A term for a dosage form in which no deliberate effort has been made to modify the drug substance release rate. The term is not used in official article titles.

Immersion: A veterinary route of administration via partial or complete submersion in a specified environment such as liquid or air.

Implant: A dosage form that is a solid or semisolid material containing the drug substance that is placed into the body. The implantation process is invasive, and the material is intended to reside at the site for a period consistent with the design release kinetics or profile of the drug substance(s).

Inhalation (By Inhalation): A route of administration for aerosols characterized by dispersion of the drug substance into the airways during inspiration.

Injection (By Injection): A route of administration of a liquid or semisolid deposited into a body cavity, fluid, or tissue by use of a needle.

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Injection: Liquid preparations that may contain drug substances and/or excipients or solutions thereof. The term "for injection" indicates dry solids that, upon the addition of a suitable vehicle, yield solutions conforming in all respects to the requirements for injections.

Injectable emulsion: Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.

Injectable suspension: Liquid preparations of solids suspended in a liquid medium. The term "for injectable suspension" indicates dry solids that, upon the addition of a suitable vehicle, yield preparations conforming in all respects to the requirements for injectable suspensions. For extended-release preparations, see *Extended-release injectable suspension*.

Insert: A solid dosage form that is inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum. It should be noted that a suppository is intended for application into the rectum and is not classified as an insert (see *Suppository*).

Intraocular: A route of administration to deliver a sterile preparation within the eye.

Irrigation: A sterile solution or liquid intended to bathe or flush open wounds or body cavities.

Jelly (not preferred; see *Gel*)**:** A semisolid dispersion of small particles or a solution of large organic molecules interpenetrated by a solution containing a gelling agent to promote stiffness.

Liposomes: A term for preparations of amphiphilic lipids that have low water solubility (see ⟨1⟩).

Liquid: A dosage form consisting of a pure chemical in its liquid state. This dosage form term should not be applied to solutions. The term is not used in official article titles. When not used in dosage form naming, the term, "liquid" is used to indicate a material that is pourable and conforms to its container at room temperature.

Lotion: An emulsion liquid dosage form applied to the outer surface of the body. Historically, this term was applied to topical suspensions and topical emulsions. The current definition of a lotion is restricted to an emulsion.

Lozenge: A solid dosage form intended to disintegrate or dissolve slowly in the mouth.

Modified-release: A term for a dosage form with a drug substance release pattern that has been deliberately changed from that observed for the immediate-release dosage form of the same drug substance. The two types of modified-release are extended-release and delayed-release. The term "modified-release" is not used in official article titles.

Molded tablet: A tablet that has been formed by dampening the ingredients and pressing into a mold, then removing and drying the resulting solid mass. This term is not used in official article titles.

Mouthwash (see *Rinse*)**:** Term applied to a solution preparation used to rinse the oral cavity. The term is not used in official article titles.

Nasal: Route of administration (mucosal) characterized by administration to the nose or by way of the nose for local or systemic effect.

Ocular (not preferred; see *Intraocular*)**:** Route of administration indicating deposition of the drug substance within the eye.

Ointment: A semisolid dosage form, usually containing less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. This dosage form generally is for external application to the skin or mucous membranes.

Ophthalmic: A route of administration characterized by application of a sterile preparation to the external parts of the eye.

Oral: Route of administration characterized by application to the mouth or delivery to the gastrointestinal tract through the mouth.

Orally disintegrating: When used in naming a dosage form, this term denotes a solid oral dosage form that disintegrates rapidly in the mouth prior to swallowing. The drug substance is intended for gastrointestinal delivery and/or absorption. See also CDER *Guidance for Industry: Orally Disintegrating Tablets*.

Orodispersible (see *Orally disintegrating*)**:** The term is not used in official article titles.

Oro-pharyngeal: A route of administration characterized by deposition of a preparation into the oral cavity and/or pharyngeal region to exert a local or systemic effect.

Otic: A route of administration characterized by deposition of a preparation into, or by way of, the ear. Sometimes referred to as *Aural* (*Aural* not preferred).

Parenteral: General route of administration which is characterized by injection through the skin or other external boundary tissue or implantation within the body. Specific parenteral routes include intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intraocular (see ⟨1⟩).

Paste: A semisolid dosage form containing a high percentage (20%–50%) of finely dispersed solids with a stiff consistency. This dosage form is intended for application to the skin, oral cavity, or mucous membranes.

Pastille (see *Lozenge*)**:** The term is not used in official article titles.

Patch (not preferred; see *System*)**:** Frequently incorrectly used to describe a *System*.

Pellet: A small solid dosage form of uniform, sometimes spherical, shape intended for direct administration. Spherical pellets are sometimes referred to as *Beads*. Pellets used in veterinary medicine are typically cylindrical in shape. Pellets intended as implants must be sterile, except for some ear implants used in animal drugs. The use of the term "pellet" for implantable dosage forms is no longer preferred (see *Implant*).

Periodontal: A term for a preparation that is applied around a tooth for localized action.

Pill: A solid, spherical dosage form usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms such as tablets or capsules.

Plaster (not preferred)**:** A dosage form containing a semisolid composition supplied on a support material for external application. Plasters are applied for prolonged periods of time to provide protection, support, or occlusion (for macerating action).

Powder: A dosage form composed of a solid or mixture of solids reduced to a finely divided state and intended for internal or external use.

Powder, inhalation: A powder containing a drug substance for oral inhalation. The powder is used with a device that aerosolizes and delivers an accurately metered amount.

Premix (not preferred; see *Animal Drugs for Use in Animal Feeds* ⟨1152⟩, *Scope, Type A Medicated Articles* and *Type B Medicated Feeds*)

Prolonged-release (see *Extended-release*)**:** The term is not used in official article titles.

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Rectal: A route of administration characterized by deposition into the rectum to provide local or systemic effect.

Rinse (see *Solution*)**:** A liquid preparation used to cleanse by flushing. A rinse is used to swish in the mouth and then expectorated. The nonpreferred term "mouthwash" has sometimes been sused for "rinse".

Semisolid: A term for a material that exhibits plastic flow behavior. A semisolid material is not pourable, does not readily conform to its container at room temperature, and does not flow at low shear stress. This term is not used in official article titles.

Shampoo: A solution, emulsion, or suspension dosage form used to clean the hair and scalp. May contain a drug substance intended for topical application to the scalp.

Soap: The alkali salt(s) of a fatty acid or mixture of fatty acids used to cleanse the skin. Soaps used as dosage forms may contain a drug substance intended for topical application to the skin. Soaps have also been used as liniments and enemas.

Soft gel capsule (not preferred; see *Capsule*)**:** A specific capsule type characterized by increased levels of plasticizers producing a more pliable and thicker-walled material than hard gelatin capsules. Soft gel capsules are further distinguished because they are single-piece sealed dosages. Frequently used for delivering liquid compositions.

Soluble tablet (see *Tablet* and *Tablet for oral solution*)**:** The term is not used in official article titles.

Solution: A clear, homogeneous liquid dosage form that contains one or more chemical substances dissolved in a solvent or mixture of mutually miscible solvents.

Spirit (not preferred; see *Solution*)**:** A liquid dosage form composed of an alcoholic or hydroalcoholic solution of volatile substances.

Spot on (Pour On): A method of delivering liquid veterinary drug products by administering them onto the animal's skin, usually between the shoulder blades (spot on) or down the back (pour on). The term is not used in official article titles.

Spray: A spray is a dosage form that contains drug substance(s) in the liquid state, either as a solution or as a suspension, and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

When not used in the naming of a dosage form, the term "spray" describes the generation of droplets of a liquid or solution to facilitate application to the intended area.

Stent, drug-eluting: A specialized form of implant used for extended local delivery of the drug substance to the immediate location of stent placement.

Strip (only used for diagnostic products, otherwise not preferred; see *Film*)**:** A dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper.

Sublingual: A route of administration characterized by placement underneath the tongue and for release of the drug substance for absorption in that region.

Suppository: A solid dosage form in which one or more drug substances are dispersed in a suitable base and molded or otherwise formed into a suitable shape for insertion into the rectum to provide local or systemic effect.

Suspension: A liquid dosage form that consists of solid particles dispersed throughout a liquid phase.

Syrup (not preferred; see *Solution*)**:** A solution containing high concentrations of sucrose or other sugars. This term is commonly used in compounding pharmacy.

System: A preparation of drug substance(s) in a carrier device that is applied topically or inserted into a body cavity. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use.

Tablet: A solid dosage form prepared from powders or granules by compaction.

Tablet for oral solution: A tablet that is intended to be dispersed in a liquid before administration. When dispersed in the liquid, a solution results.

Tablet for oral suspension: A tablet that is intended to be dispersed in a liquid before administration. When dispersed in the liquid, a suspension results.

Tape (not preferred)**:** A dosage form or device composed of a woven fabric or synthetic material onto which a drug substance is placed, usually with an adhesive on one or both sides to facilitate topical application. The rate of release of the drug substance is not controlled.

Tincture (not preferred; see *Solution*)**:** An alcoholic or hydroalcoholic solution prepared from vegetable materials or from chemical substances.

Topical: A route of administration characterized by application to the external surface of the body.

Transdermal: A route of administration characterized by drug product application to the skin where the drug substance passes through the dermal layer with the intent to achieve a systemic effect.

Troche (see *Lozenge*)**:** A solid dosage form intended to disintegrate or dissolve slowly in the mouth and usually prepared by compaction in a manner similar to that used for tablets. The term is not used in official article titles.

Urethral: A route of administration characterized by deposition into the urethra.

Vaginal: A route of administration characterized by deposition into the vagina.

Vehicle: A term commonly encountered in compounding pharmacy that refers to a component for internal or external use that is used as a carrier or diluent in which liquids, semisolids, or solids are dissolved or suspended. Examples include water, syrups, elixirs, oleaginous liquids, solid and semisolid carriers, and proprietary products (see *Excipient*). This term is not used in official article titles.

Veterinary: A term for dosage forms intended for nonhuman use.

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⟨1152⟩ ANIMAL DRUGS FOR USE IN ANIMAL FEEDS

PURPOSE

This chapter provides general descriptions of, and definitions for, animal drugs and drug products delivered in animal feeds. It discusses general principles involved in the manufacture, packaging, and labeling of these drugs and drug products.

SCOPE

Medicated articles and feeds that are used to deliver animal drug(s) via the food given to animals are discussed in this general information chapter. Drugs approved for further manufacture into medicated animal feeds are not dosage form drugs. Dosage form drugs administered with feeds are not medicated articles or feeds. Drug dosage forms are listed in *USP* general chapter *Pharmaceutical Dosage Forms* ⟨1151⟩.

Animal drugs approved for further manufacture into medicated animal feeds may be in either dry or liquid form. They are sometimes referred to as premixes. The term "premix" is no longer used for animal drugs for use in animal feeds but is still used in some older drug monographs. Animal drugs in feeds are regulated as Type A medicated articles and Type B and Type C medicated feeds.

Type A Medicated Articles

Type A medicated articles [21 CFR 558.3(b)(2)] are concentrated forms of animal drugs intended solely for further manufacture of other approved Type A medicated articles or Type B or C medicated feeds. This means that Type A medicated articles cannot be fed directly to animals. They consist of one or more animal drug(s) with or without a carrier (e.g., calcium carbonate, rice hull, corn, gluten) and with or without other inactive ingredients. They can be prepared in dry or liquid form. They are sold to feed mills or livestock producers and are intended to be further diluted by mixing into feed before consumption by animals.

Type B Medicated Feeds

Type B medicated feeds [21 CFR 558.3(b)(3)] are intermediate medicated feeds for animals. They are manufactured from either Type A medicated article(s) or other Type B medicated feed(s) by dilution with non-medicated feed ingredients. In addition to the animal drug(s), Type B medicated feeds contain a substantial quantity of nutrients that comprise NLT 25% of the total feed weight. They can be prepared in dry or liquid form. Similar to Type A medicated articles, Type B medicated feeds are intended only for further dilution by mixing into feed, and they are not approved for feeding to animals.

Type C Medicated Feeds

Type C medicated feeds [21 CFR 558.3(b)(4)] are intended to be fed directly to animals. They are manufactured from Type A medicated articles, Type B medicated feeds, or other Type C medicated feeds diluted with non-medicated feed ingredients. Type C medicated feeds may be prepared in dry or liquid form. Type C medicated feeds can be either fed as the complete feed for the animals, top-dressed onto the animals' normal daily rations, or offered "free-choice" (21 CFR 510.455). Type C medicated feeds approved to be offered free-choice are not intended to be fully consumed in a single feeding or to constitute the entire diet of the animals.

PREPARATION

Type A medicated articles in dry form are typically produced by blending the drug substance(s) with carriers and other excipients to promote uniform mixing when subsequently added to the animal feed. The drug substance(s) may first be mixed with an excipient (e.g., starch or sodium aluminosilicate) that has a similar particle size and can help distribute the drug substance(s) uniformly throughout the final mixture. This pre-blend may then be mixed with bulk excipients (e.g., calcium carbonate or soybean hulls). The product may be granulated and/or oil (e.g., mineral oil, soybean oil) may be added to aid uniform distribution, to prevent particle segregation during shipping, and/or to minimize formation of airborne drug substance particles during production of another Type A medicated article or Types B or C medicated feeds.

Type A medicated articles in liquid form are produced by mixing the drug substance(s) with a suitable solvent (e.g., water or propylene glycol). The drug substance(s) is usually dissolved to produce a solution, but suspension products could be produced also.

Types B or C medicated feeds are typically manufactured at feed mills or on-farm by livestock producers. To manufacture Types B or C medicated feeds in dry form, Type A medicated articles are added to the feeds during the mixing process. Liquid Type A medicated articles may be sprayed in at set rates, and dry Type A medicated articles are added using methods that facilitate uniform distribution in the feeds. Types B and C medicated feeds in dry form may be further processed by heating, steaming, and extruding into pellets. The pellets may be rolled or broken up to create crumbles. Types B and C medicated feeds may also be prepared in liquid form. Liquid feeds are typically molasses based and contain an animal drug(s) dissolved or suspended in the liquid matrix. The liquid feed may need to be recirculated or agitated on a routine basis to maintain a uniform distribution of the drug(s).

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A Type B medicated feed may also be prepared by diluting another Type B medicated feed. A Type C medicated feed may also be prepared by diluting a Type B medicated feed or another Type C medicated feed.

LABELING AND PACKAGING

Labeling for Type A medicated articles and Types B and C medicated feeds provides all information necessary for their safe and effective use. The label for a Type A medicated article includes mixing directions for the manufacture of medicated feeds from the Type A medicated article and feeding directions for the Type C medicated feeds. The label for a Type B medicated feed provides mixing directions for the manufacture of medicated animal feeds. Labels for both Type A medicated articles and Type B medicated feeds indicate that they are not to be fed directly to animals. The label for Type C medicated feeds includes directions for feeding.

Type A medicated articles are packaged in bags (e.g., paper with polyethylene liners) for dry products or in appropriate containers (e.g., plastic) for liquids. Typical sizes are 50-lb bags or several-gallon containers. Dry Types B and C medicated feeds may be packaged in bags for storage and delivery, or they may be shipped in bulk form for storage or immediate use. Free-choice Type C medicated feeds may be packaged in bags (e.g., loose minerals), wrapped in film (e.g., compressed blocks), or packaged in tubs (e.g., molded blocks). Liquid Types B and C medicated feeds are stored and shipped in bulk in tanks.

NOMENCLATURE

The drug product's established (non-proprietary) name consists of the drug substance (active moiety) and an appropriate type of medicated article or feed. The following nomenclature options are available to indicate types of medicated articles or feeds as part of the product established name:

[Drug] Type A medicated article
[Drug] Type A liquid medicated article
[Drug] Type B medicated feed
[Drug] Type B liquid medicated feed
[Drug] Type C medicated feed
[Drug] Type C liquid medicated feed
[Drug] Type C free-choice medicated feed
[Drug] Type C liquid free-choice medicated feed
[Drug] Type C top-dress medicated feed

⟨1160⟩ PHARMACEUTICAL CALCULATIONS IN PHARMACY PRACTICE

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1. INTRODUCTION

The purpose of this general chapter is to provide general information to assist pharmacists and support personnel in performing the necessary calculations for compounding and dispensing medications. This general chapter is not inclusive of all the information necessary for performing pharmaceutical calculations. For additional information regarding pharmaceutical calculations, consult a pharmaceutical calculations textbook. For additional information on pharmaceutical compounding and drug stability, see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, *Packaging and Storage Requirements* ⟨659⟩, *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩, and *Stability Considerations in Dispensing Practice* ⟨1191⟩.

Correct pharmaceutical calculations can be accomplished by using proper conversions from one measurement system to another and properly placing decimal points (or commas, in countries where it is customary to use these in the place of decimal points), by understanding the arithmetical concepts, and by paying close attention to the details of the calculations. Before proceeding with any calculation, pharmacists should do the following: (a) read the entire formula or prescription carefully; (b) determine the materials that are needed; and then (c) select the appropriate methods of preparation and the appropriate calculations.

Logical methods that require as few steps as possible should be selected to ensure that calculations are done accurately and correctly. A pharmacist should double-check each calculation or have someone else double-check, e.g., a technician, if another pharmacist is not available, before proceeding with compounding the preparation. One expedient method of double-checking is estimation, which consists of convenient rounding (e.g., 0.012 to 0.01, 0.44 to 0.5, 18.3 to 20, and 476 to 500) to approximate the magnitude of answers.

Change to read:

2. ▲▲ (USP 1-MAY-2019) AMOUNTS OF ACTIVE INGREDIENTS

The pharmacist must be able to calculate the amount or concentration of drug substances in each unit or dosage portion of a compounded preparation at the time it is prepared and again at the time it is dispensed. Pharmacists must perform calculations and measurements to obtain, theoretically, 100% of the amount of each ingredient in compounded formulations. Calculations must account for the active ingredient, or active moiety, and water content of drug substances, which includes those in the chemical formulas of hydrates. Official drug substances and added substances must meet the requirements in general chapter *Loss on Drying* ⟨731⟩, which must be included in the calculations of amounts and concentrations of ingredients. The pharmacist should consider the effect of ambient humidity on the gain or loss of water from drugs and added substances in containers subjected to intermittent opening over prolonged storage. Each container should be opened for the shortest duration necessary and then closed tightly immediately after use.

The nature of the drug substance to be weighed and used in compounding a prescription must be known. If the substance is a hydrate, its anhydrous equivalent weight may need to be calculated. On the other hand, if there is adsorbed moisture present that is either specified on a Certificate of Analysis (CoA) or that is determined in the pharmacy immediately before the drug substance is used in the preparation (see ⟨731⟩), this information must be used when calculating the amount of drug substance that is to be weighed to determine the exact amount of anhydrous drug substance required.

There are cases in which the required amount of a dose is specified in terms of a cation (e.g., Li^+), an anion (e.g., F^-), or a molecule (e.g., theophylline in aminophylline). In these instances, the drug substance weighed is a salt or complex, a portion of which represents the pharmacologically active moiety. Thus, the exact amount of such substances weighed must be calculated on the basis of the required quantity of the pharmacological moiety.

The following formula may be used to calculate the theoretical weight of an ingredient in a compounded preparation:

$$W = AB/CD$$

W = actual weighed amount
A = prescribed or pharmacist-determined weight of the active or functional moiety of drug or added substance
B = molecular weight (MW) of the ingredient, including waters of hydration for hydrous ingredients
C = MW of the active or functional moiety of a drug or added substance that is provided in the MW of the weighed ingredient
D = the fraction of dry weight when the percent by weight of adsorbed moisture content is known from the loss on drying procedure (see ⟨731⟩) or from the CoA. The CoA should be lot specific.

2.1 Active Ingredients

2.1.1 CALCULATING DRUGS DOSED AS SALT FORM AND HYDRATE

Examples—Drugs dosed as salt form and hydrate

1. Drugs dosed as salt form and hydrate

Triturate morphine sulfate and lactose to obtain 10 g in which there are 30 mg of morphine sulfate for each 200 mg of the morphine–lactose mixture. [NOTE—Morphine is dosed as the morphine sulfate, which is the pentahydrate.]

W = weight of morphine sulfate (g)
A = weight of morphine sulfate pentahydrate in the prescription, 1.5 g
B = MW of morphine sulfate pentahydrate, 759 g/mol
C = MW of morphine sulfate pentahydrate, 759 g/mol

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D = 1.0

To solve the equation:

$$W = (1.5\ g \times 759\ g/mol)/(759\ g/mol \times 1) = 1.5\ g\ of\ morphine\ sulfate\ pentahydrate$$

2. Active drug moiety and correction for moisture

Accurately weigh an amount of aminophylline to obtain 250 mg of anhydrous theophylline. [NOTE—In this example, the powdered aminophylline dihydrate weighed contains 0.4% w/w absorbed moisture as stated in the CoA received by the pharmacy.]

$$W = AB/CD$$

W = weight of aminophylline dihydrate (mg)
A = weight of anhydrous theophylline, 250 mg
B = MW of aminophylline dihydrate, 456 g/mol
C = MW of anhydrous theophylline, 360 g/mol
D = 0.996

[NOTE—One mol of aminophylline contains 2 mol of theophylline. Theophylline has a MW of 180.]

To solve the equation:

$$W = (250\ mg \times 456\ g/mol)/(360\ g/mol \times 0.996) = 318\ mg\ of\ aminophylline\ dihydrate$$

2.2 Hydrates, Salts, and Esters

Frequently, for stability or other reasons such as taste or solubility, the base form of a drug is administered in another form such as a salt or an ester. This altered form of the drug usually has a different MW, and at times it may be useful to determine the amount of the base form of the drug in the altered form.

2.2.1 CALCULATING HYDRATES, SALTS, AND ESTERS

Examples—Hydrates, salts, and esters

1. Hydrates

If a prescription for 100 g of lidocaine hydrochloride 2% gel is to be made, 2 g of anhydrous lidocaine hydrochloride could be used, or the equivalent amount of lidocaine hydrochloride monohydrate could be calculated as follows:

W = weight of lidocaine hydrochloride monohydrate (g)
A = weight of anhydrous lidocaine hydrochloride in the prescription, 2 g
B = MW of lidocaine hydrochloride monohydrate, 288.81 g/mol
C = MW of anhydrous lidocaine hydrochloride, 270.80 g/mol
D = 1.0

To solve the equation:

$$W = (2\ g \times 288.81\ g/mol)/(270.80\ g/mol \times 1) = 2.133\ g\ of\ lidocaine\ hydrochloride\ monohydrate$$

2. Salts

A prescription calls for 10 mL of a fentanyl topical gel at a concentration 50 mcg fentanyl/0.1 mL prepared from fentanyl citrate. The amount of fentanyl citrate required for the preparation could be calculated as follows:

Amount of fentanyl needed for the preparation:

$$(50\ mcg\ fentanyl/0.1\ mL) \times 10\ mL = 5000\ mcg\ of\ fentanyl$$

W = weight of fentanyl citrate in the prescription (mcg)
A = weight of fentanyl in the prescription, 5000 mcg
B = MW of fentanyl citrate, 528.59 g/mol
C = MW of fentanyl, 336.47 g/mol
D = 1.0

To solve the equation:

$$W = (5000\ mcg \times 528.59\ g/mol)/(336.47\ g/mol \times 1) = 7855\ mcg\ of\ fentanyl\ citrate$$

3. Esters

The amount of cefuroxime axetil contained in a single 250-mg cefuroxime tablet can be calculated as follows:

W = weight of cefuroxime axetil in tablet (mg)
A = weight of cefuroxime in the prescription, 250 mg
B = MW of cefuroxime axetil, 510.47 mg/mmol
C = MW of cefuroxime, 424.39 mg/mmol
D = 1.0

To solve the equation:

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W = (250 mg × 510.47 g/mol)/(424.39 g/mol × 1) = 300 mg of cefuroxime axetil

Change to read:

3. DOSAGES ▲BY BODY WEIGHT AND SURFACE AREA▲ (USP 1-MAY-2019)

3.1 Dosing by Weight

Doses are frequently expressed as mg of drug per kg of body weight per a dosing interval.

3.1.1 CALCULATING DOSING BY WEIGHT

Example—Dosing by weight

A physician orders azithromycin for oral suspension at a dose of 15 mg/kg/day, divided every 12 h, for a child that weighs 36 lb. Calculate the volume of oral suspension, in mL, that should be administered for each dose of a 200-mg/5-mL suspension as follows:

1. Calculate the child's weight in kg:

$$36\ \text{lb} \times \text{kg}/2.2\ \text{lb} = 16.4\ \text{kg}$$

2. Multiply the weight, in kg, by the dosing rate:

$$16.4\ \text{kg} \times 15\ \text{mg/kg/day} = 246\ \text{mg/day}$$

3. Divide the total daily dose by the number of doses/day:

$$246\ \text{mg}/2\ \text{doses} = 123\ \text{mg/dose}$$

4. Calculate the volume of each dose using ratio and proportion:

$$(123\ \text{mg/dose})/(200\ \text{mg}/5\ \text{mL}) = 3.1\ \text{mL/dose}$$

Some calculations may also be completed using dimensional units analysis (DUA). The DUA should begin at the left end with a factor containing the numerator answer units. All units other than those in the answer should cancel. If using DUA, the preceding equation would be as follows:

$$36\text{-lb child} \times \frac{\text{kg}}{2.2\ \text{lb}} \times \frac{15\ \text{mg}}{\text{kg} \cdot \text{day}} \times \frac{5\ \text{mL}}{200\ \text{mg}} \times \frac{\text{day}}{2\ \text{doses}} = \frac{3.1\ \text{mL}}{\text{dose}}$$

3.2 Dosing by Body Surface Area (Humans)

Some medications, including chemotherapeutic agents, require dosing by body surface area (BSA). The dose is expressed as amount of drug per meter squared (m^2). BSA may be calculated using the following formulas:

$$\text{BSA}\ (m^2) = \sqrt{[\text{Height (in)} \times \text{Weight (lb)}]/3131}$$

$$\text{BSA}\ (m^2) = \sqrt{[\text{Height (cm)} \times \text{Weight (kg)}]/3600}$$

3.2.1 CALCULATING BY BODY SURFACE AREA (HUMAN)

Example—Dosing by BSA (humans)

A physician orders rituximab at a dose of 375 mg/m^2 every week for 6 weeks for a patient who is 6 ft 2 in tall and weighs 183 lb. Calculate the volume, in mL, of 10-mg/mL rituximab injection needed to make each IV infusion dose as follows:

1. Calculate the patient's BSA:

$$m^2 = \sqrt{[74\ \text{in} \times 183\ \text{lb}]/3131} = 2.08\ m^2$$

2. Multiply the BSA by the dosing rate:

$$2.08\ m^2 \times 375\ \text{mg}/m^2 = 780\ \text{mg/dose}$$

3. Calculate the volume of each dose using ratio and proportion:

$$(780\ \text{mg/dose})/(10\ \text{mg/mL}) = 78\ \text{mL/dose}$$

The preceding calculation may also be completed using DUA as follows:

$$2.08\text{-}m^2\ \text{patient} \times \frac{375\ \text{mg}}{m^2 \cdot \text{dose}} \times \frac{\text{mL}}{10\ \text{mg}} = \frac{78\ \text{mL}}{\text{dose}}$$

3.3 Dosing By Body Surface Area (Animals)

BSA for cats and dogs may be calculated using the following formulas. For other animals, consult an appropriate veterinary medicine reference.

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Body surface area for cats:

$$BSA\ (m^2) = \{10 \times [body\ weight\ (g)]^{0.667}\}/10{,}000$$

Body surface area for dogs:

$$BSA\ (m^2) = \{10.1 \times [body\ weight\ (g)]^{0.667}\}/10{,}000$$

3.3.1 CALCULATING DOSING BY BODY SURFACE AREA (ANIMALS)

Example—Dosing by BSA (animals)
A veterinarian orders oral cyclophosphamide therapy at a dose of 50 mg/m² for a cat who weighs 5.8 kg. Calculate the dose of cyclophosphamide as follows:

1. Calculate the cat's BSA:

$$BSA\ (m^2) = [10 \times (5800\ g)^{0.667}]/10{,}000 = 0.324\ m^2$$

2. Multiply the BSA by the dosing rate:

$$0.324\ m^2 \times 50\ mg/m^2 = 16.2\ mg$$

Change to read:

4. ▲BIOLOGICAL▲ (USP 1-MAY-2019) POTENCY UNITS

Because some substances cannot be completely characterized by chemical and physical means, it may be necessary to express quantities of activity in biological units of potency [see the *USP General Notices 5.50.10, Units of Potency (Biological)*].

4.1 Calculating by Use of Potency Units

EXAMPLES—USE OF POTENCY UNITS

1. Potency units-to-milligrams conversion
A dose of penicillin G benzathine for streptococcal infection is 1.2 million units administered intramuscularly. If a specific product contains 1180 units/mg, calculate the amount, in mg, of penicillin G benzathine in the dose as follows:

$$(1{,}200{,}000\ units)/(1180\ units/mg) = 1017\ mg\ of\ penicillin\ G\ benzathine$$

2. Potency units-to-milligrams conversion
A prescription calls for 60 g of an ointment containing 150,000 units of nystatin per gram. Calculate the quantity of nystatin with a potency of 4400 units/mg that should be weighed for the prescription as follows:

$$60\ g \times (150{,}000\ units\ of\ nystatin/g) = 9{,}000{,}000\ units$$

$$9{,}000{,}000\ units/(4400\ units/mg) = 2045\ mg\ of\ nystatin$$

5. VOLUME AND WEIGHT SUMS

Weights are additive in most mixtures of liquids, semisolids, and solids. Volumes in mixtures of miscible solutions and pure liquids may or may not be additive, based primarily on the effects of volume proportions and intermolecular hydrogen bonding. For example, mixtures containing equal or near-equal volumes of water and ethanol (and other miscible mono-hydroxy alcohols) will be exothermic and result in a volume contraction of <5%, e.g., 50 mL of water + 50 mL of ethanol yield 97–98 mL at 20°–25°. Negligible volume contraction occurs between water and polyhydroxy or polyhydric alcohols, e.g., glycerin and propylene glycol. Volumes are additive with usually negligible error in aqueous mixtures that contain <10% of mono-hydroxy alcohols, i.e., there is <0.5% volume contraction.

6. DENSITY AND SPECIFIC GRAVITY

Density is defined as the mass of a substance in air at a specific temperature (typically 25°) per unit volume of that substance at the same temperature. Density may be calculated with the following equation:

$$Density = (mass\ of\ substance/volume\ of\ substance)\ at\ a\ particular\ temperature\ and\ pressure$$

Specific gravity (SG) is the unitless ratio of the density of a substance to the density of water at 4°, or [(g of substance/mL)/1.00 g/mL]. Alternatively, SG can be calculated at a particular temperature in some common units of density from density of substance per density of water.
SG may be calculated with the following equation:

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SG = (weight of the substance)/(weight of an equal volume of water)

6.1 Calculating Density and Specific Gravity

EXAMPLES—DENSITY AND SPECIFIC GRAVITY

1. Density calculation
2.3 g of activated charcoal powder occupies a bulk volume of 5.2 mL at 20° and 1 atm. The density of activated charcoal powder can be calculated as follows:

$$\text{Density} = 2.3\ \text{g}/5.2\ \text{mL} = 0.44\ \text{g/mL}$$

2. SG calculation
125 g of glycerin occupies a volume of 99 mL at 25°. [NOTE—The density of water at 25° is 0.997 g/mL.] The SG of glycerin can be calculated as follows:

$$\text{SG} = (125\ \text{g}/99\ \text{mL})/(0.997\ \text{g/mL}) = 1.266$$

3. Concentrated acid calculation
Hydrochloric acid is approximately a 37% w/w solution of hydrochloric acid in water. Calculate the amount, in g, of hydrochloric acid contained in 75 mL of hydrochloric acid as follows. [NOTE—The SG of hydrochloric acid is 1.18.]

$$37\%\ \text{w/w} \times 1.18 = 43.7\%\ \text{w/v}$$

$$(43.7\ \text{g}/100\ \text{mL}) \times 75\ \text{mL} = 32.8\ \text{g of hydrochloric acid}$$

7. MILLIEQUIVALENTS AND MILLIMOLES

[NOTE—This section addresses milliequivalents (mEq) and millimoles (mmol) as they apply to electrolytes for dosage calculations. See also *8. Concentration Expressions*.]
The quantities of electrolytes administered to patients are usually expressed in terms of mEq. Weight units such as mg or g are not often used for electrolytes because the electrical properties of ions are best expressed as mEq. An equivalent (Eq) is the weight of a substance that supplies 1 unit of charge. An equivalent weight is the weight, in g, of an atom or radical, divided by the valence of the atom or radical. A mEq is $1/1000^{th}$ of an Eq. The equivalent weight of a compound may be determined by dividing its formula or MW in g by the valence of its largest valence ion.
A mole equals one gram-atomic weight or gram-molecular weight of a substance. A millimole equals $1/1000^{th}$ of a mole.

7.1 Calculating Milliequivalents and Millimoles

EXAMPLES—MILLIEQUIVALENTS AND MILLIMOLES

1. Calculate the mEq weight of calcium. [NOTE—Calcium has a MW of 40.08, and the valence of calcium is 2^+.]

$$\text{Eq weight} = 40.08\ \text{g}/2 = 20.04\ \text{g}$$

$$\text{mEq weight} = 20.04\ \text{g}/1000 = 0.02004\ \text{g} = 20.04\ \text{mg}$$

2. Calculate the quantity, in mEq, of potassium in a 250-mg Penicillin V Potassium Tablet. [NOTE—Penicillin V potassium has a MW of 388.48 g, there is one potassium atom in the molecule, and the valence of potassium is 1^+.]

$$\text{Eq weight} = 388.48\ \text{g}/1 = 388.48\ \text{g}$$

$$\text{mEq weight} = 388.48\ \text{g}/1000 = 0.38848\ \text{g} = 388.48\ \text{mg}$$

$$(250\ \text{mg/tablet})/(388.48\ \text{mg/mEq}) = 0.644\ \text{mEq of potassium/tablet}$$

3. Calculate the mEq of magnesium and sulfate in a 2-mL dose of 50% Magnesium Sulfate Injection. [NOTE—Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) has a MW of 246.47, and the highest valence ion is magnesium 2^+ and sulfate 2^-.]

$$(50\ \text{g}/100\ \text{mL}) \times (2\ \text{mL/dose}) = 1\ \text{g/dose}$$

$$\text{Eq weight} = 246.47\ \text{g}/2 = 123.24\ \text{g/Eq}$$

$$(1\text{g/dose})/(123.24\ \text{g/Eq}) = 0.008114\ \text{Eq} = 8.114\ \text{mEq of both magnesium and sulfate per dose}$$

This problem may also be worked using DUA as follows:

$$\frac{50\ \text{g}}{100\ \text{mL}} \times \frac{2\ \text{mL}}{\text{dose}} \times \frac{2\ \text{Eq}}{246.47\ \text{g}} \times \frac{1000\ \text{mEq}}{\text{Eq}} = \frac{8.114\ \text{mEq}}{\text{dose}}$$

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4. A vial of sodium chloride injection contains 3 mEq/mL of sodium chloride. Calculate the strength, in % w/v, of the injection. [NOTE—Sodium chloride has a MW of 58.44.]

$$\frac{3\ \text{mEq}}{\text{mL}} \times \frac{58.44\ \text{g}}{1\ \text{Eq}} \times \frac{\text{Eq}}{1000\ \text{mEq}} = \frac{0.1753\ \text{g}}{\text{mL}}$$

(0.1753 g/mL) × 100 mL = 17.53 g in 100 mL = 17.53% w/v

5. Calculate the weight of potassium in mmol. [NOTE—Potassium has a MW of 39.1.]
The weight of 1 mol is 39.1 g and the weight in mmol is:

39.1 g/1000 = 0.0391 g or 39.1 mg

6. Calculate the mmol of penicillin V potassium in a 250-mg Penicillin V Potassium Tablet. [NOTE—Penicillin V potassium has a MW of 388.48.]
The weight of 1 mol is 388.48 g, and the weight of 1 mmol is:

388.48 g/1000 = 0.38848 g or 388.48 mg

$$\frac{250\ \text{mg}}{\text{tablet}} \times \frac{\text{mmol}}{388.48\ \text{mg}} = \frac{0.644\ \text{mmol of Penicillin V potassium}}{\text{tablet}}$$

8. CONCENTRATION EXPRESSIONS

The concentration expressions in this section refer to homogeneous mixtures of the following states of matter at a temperature of 20°–30° and pressure of 1 atm (29.92 in Hg, 760 mm Hg, 101.3 kPa, 1013.3 mb): gas in gas, gas in liquid, liquid in liquid, liquid in semisolid, solid in liquid, solid in semisolid, and solid in solid. Concentration expressions used in pharmacy practice and pharmaceutical research include, but are not limited to, those listed in *Table 1*. Common metric drug strength and clinical concentrations include, for example, mcg/mL, mg/dL, g or mg per L, and ng/µL (see *General Notices 8.240, Weights and Measures*).

Table 1

Title	Abbreviation	Definition
Mass in volume ratios	None is standard	Mass of a dispersed or dissolved ingredient per volume amount of mixtures containing that ingredient
mEq[a] per volume	mEq/volume unit	mEq of an electrolyte or salt per unit of volume of solutions containing that electrolyte or salt
Molality	m	mol[b] of a solute/kg of a solvent containing that solute[c]
Molarity	M	mol of a solute/L of a solvent containing that solute[d]
Normality[e]	N	Equivalents (Eq[f]) of a solute/L of a solvent containing that solute[g]
Parts per million	ppm	Parts of a gas, liquid, or solid per 1 million part of another gas, liquid, or solid containing the first gas, liquid, or solid
% Volume in volume	% v/v	mL of liquid per 100 mL of a solvent containing that liquid
% Weight in volume	% w/v	g of a solute per 100 mL of a solvent containing that solute
% Weight in weight	% w/w	g of a solute per 100 g of a mixture containing that solute
Ratio strength	1:*R*	1 part of an ingredient per R^h parts of a mixture containing that ingredient
	1 in *R*	1 part of an ingredient in R^h parts of a mixture containing that ingredient
	X:*Y*	X^h parts of one ingredient per Y^h parts of another ingredient in a mixture

[a] 1 mEq = Eq/1000.
[b] The abbreviation for mole is mol.
[c] 1 mol of solute per 1 kg of solvent is a 1 molal (1 m) solution.
[d] 1 mol of solute per 1 L of solution of that solute is a 1 molar (1 M) solution.
[e] Normality = (Molarity × largest valence ion of a compound), e.g., (18 M H_2SO_4 × 2) = 36 N H_2SO_4, where 2 derives from the 2^- valence of SO_4.
[f] Eq of a compound = (1 mol × largest valence ion of a compound), e.g., 1 mol of lithium citrate = 3 Eq of lithium citrate; 1 mol of $Ca(gluconate)_2$ = 2 Eq of $Ca(gluconate)_2$; and 1 mol of KCl = 1 Eq of KCl.
[g] 1 Eq of solute per 1 L of solution of that solute is a 1 normal (1 N) solution.
[h] *R*, *X*, and *Y* are whole numbers.

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8.1 Calculating Normality

EXAMPLE—NORMALITY

Calculate the amount of sodium bicarbonate powder needed to prepare 50 mL of a 0.07 N solution of sodium bicarbonate ($NaHCO_3$). [NOTE—Sodium bicarbonate has a MW of 84.01.] In an acid or base reaction, because $NaHCO_3$ may act as an acid by giving up one proton, or as a base by accepting one proton, one Eq of $NaHCO_3$ is contained in each mole of $NaHCO_3$.

$$0.050\ \text{L} \times \frac{0.07\ \text{Eq}}{\text{L}} \times \frac{1\ \text{mol}}{1\ \text{Eq}} \times \frac{84.01\ \text{g}}{1\ \text{mol}} = 0.294\ \text{g of sodium bicarbonate}$$

8.2 Calculating Percentage Concentrations

Percentage concentrations of solutions and other homogeneous mixtures are usually expressed in one of three common forms in which numerator and denominator quantities are in g and mL measurement units.

1. Volume percent (% v/v) = (volume of liquid solute/volume of solution or suspension) × 100
   or % v/v = mL of liquid solute in 100 mL of solution or suspension
2. Weight percent (% w/w) = (weight of solute/weight of mixture) × 100
   or % w/w = g of ingredient in 100 g of mixture
3. Weight in volume percent (% w/v) = (weight of solute/volume of solution or suspension) × 100
   or % w/v = g of solute in 100 mL of solution or suspension

The preceding three equations may be used to calculate any one of the three values (i.e., weights, volumes, or percentages) in a given equation if the other two values are known (see also *General Notices 8.140, Percentage Concentrations*).

EXAMPLES—PERCENTAGE CONCENTRATIONS

1. Weight percent
   A prescription order reads as follows (see *Table 2*):

Table 2

Zinc oxide	7.5 g
Calamine	7.5 g
Starch	15 g
White petrolatum	30 g

Calculate the percentage concentration for each of the four components using the preceding weight percent equation as follows:

A. The total weight of ointment = 7.5 g + 7.5 g + 15 g + 30 g = 60.0 g
B. The weight percent of zinc oxide = (7.5 g of zinc oxide/60 g of ointment) × 100% = 12.5%
C. The weight percent of calamine = (7.5 g of calamine/60 g of ointment) × 100% = 12.5%
D. The weight percent of starch = (15 g of starch/60 g of ointment) × 100% = 25%
E. The weight percent of white petrolatum = (30 g of white petrolatum/60 g of ointment) × 100% = 50%

2. Volume percent
   A prescription order reads as follows:
   Rx: Eucalyptus Oil 3% v/v in Mineral Oil.
   Dispense 30 mL.
   Calculate the quantities of ingredients in this prescription using the volume percent equation as follows:
   A. The amount of eucalyptus oil.

$$3\%\ \text{v/v} = (\text{volume of oil in mL}/30.0\ \text{mL}) \times 100\%$$

$$\text{volume in oil} = 0.9\ \text{mL of eucalyptus oil}$$

   B. The amount of mineral oil.

$$30\ \text{mL} - 0.9\ \text{mL} = 29.1\ \text{mL of mineral oil}$$

8.3 Conversions of Concentration Expressions

8.3.1 SOLID-IN-LIQUID SOLUTION CONVERSIONS

The calculations used to convert from percent weight in volume, % w/v, to other concentrations and vice versa, using the same densities and formula or MWs, are illustrated as follows for calcium chloride, magnesium sulfate, and potassium chloride solutions in water.

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8.3.1.1 Calculating solid-in-liquid conversions

Examples—Solid-in-liquid conversions

1. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to molality (m). [NOTE—Calcium chloride has a MW of 147.01 g; 10% w/v solution has a density of 1.087 g/mL.]

10% w/v = 10 g of calcium/100 mL of solution

Using the density of the solution:

100 mL of solution × 1.087 g/mL = 108.7 g of solution

108.7 g of solution – 10 g of calcium chloride = 98.7 g of water = 0.0987 kg of water

10 g of calcium chloride/(147.01 g of calcium chloride/mol of calcium chloride) = 0.068 mol of calcium chloride

0.068 mol of calcium chloride/0.0987 kg of water = 0.689 m

2. Convert 50% w/v magnesium sulfate ($MgSO_4 \cdot 7H_2O$) to molarity (M). [NOTE—Magnesium sulfate has a MW of 246.47 g.]

$$\frac{50\ \text{g}}{100\ \text{mL}} \times \frac{\text{mol}}{246.47\ \text{g}} \times \frac{1000\ \text{mL}}{1\ \text{L}} = 2.029\ \text{M}$$

3. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to normality (N).

$$\frac{10\ \text{g}}{100\ \text{mL}} \times \frac{1\ \text{mol}}{147.01\ \text{g}} \times \frac{2\ \text{Eq}}{1\ \text{mol}} \times \frac{1000\ \text{mL}}{1\ \text{L}} = 1.36\ \text{N}$$

*2 Eq/mol derived from the 2^+ valence of calcium

4. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to mEq/mL.

$$\frac{10\ \text{g}}{100\ \text{ml}} \times \frac{1\ \text{mol}}{147.01\ \text{g}} \times \frac{2\ \text{Eq}}{1\ \text{mol}} \times \frac{1000\ \text{mEq}}{1\ \text{Eq}} = 1.36\ \text{mEq/mL}$$

5. Convert 0.1% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to ppm.

(0.1 g/100 mL) × (1×10^6 ppm) = 1000 ppm

6. Convert 33% w/v potassium chloride (KCl) to 1:*R* ratio strength.

(1/*R*) = (33 g/100 mL)

R = 3.03

1:*R* = 1:3

8.3.2 LIQUID-IN-LIQUID SOLUTION CONVERSIONS

The calculations used to convert from percent weight in weight, % w/w, and volume in volume, % v/v, to other concentrations and vice versa using the same densities and formula or MWs, are illustrated for glycerin and isopropyl alcohol in water. Besides liquid-in-semisolid, solid-in-semisolid, and solid-in-solid mixtures, % w/w is used for viscous liquids, such as coal tar, glycerin, and concentrated acids.

8.3.2.1 Converting liquid-in-liquid solutions

Examples—Liquid-in-liquid conversions

1. Convert 50% w/w glycerin to % w/v. [NOTE—50% w/w glycerin has a density of 1.13 g/mL.]

(50 g/100 g) × (1.13 g/mL) = 0.565 g/mL

56.5 g/100 mL = 56.5% w/v

2. Convert 70% v/v isopropyl alcohol to % w/w. [NOTE—Isopropyl alcohol has a density of 0.79 g/mL, and 70% v/v isopropyl alcohol has a density of 0.85 g/mL.]

70 mL of isopropyl alcohol × (0.79 g/mL) = 55.3 g of isopropyl alcohol

100 mL of solution × (0.85 g/mL) = 85 g of solution

(55.3 g of isopropyl alcohol/85 g of solution) × 100 = 65.06% w/w

3. Convert 70% v/v isopropyl alcohol to % w/v. The following values are from example 2.

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55.3 g of isopropyl alcohol/100 mL of solution = 55.3% w/v

4. Convert 50% w/w glycerin to molality (m). [NOTE—Glycerin has a MW of 92.1.]

50 g of glycerin/(92.1 g/mol) = 0.543 mol of glycerin

100 g of solution – 50 g of glycerin = 50 g of water = 0.05 kg of water

(0.543 mol of glycerin/0.05 kg of water) = 10.86 m

5. Convert 70% v/v isopropyl alcohol to molality (m). [NOTE—Isopropyl alcohol has a density of 0.79 g/mL and a MW of 60.1; 70% v/v isopropyl alcohol has a density of 0.85 g/mL.]

70 mL of isopropyl alcohol × (0.79 g/mL) = 55.3 g of isopropyl alcohol

100 mL of solution × (0.85 g/mL) = 85 g of solution

(85 g of solution – 55.3 g of isopropyl alcohol) = 29.7 g of water = 0.0297 kg of water

55.3 g of isopropyl alcohol/(60.1 g/mol) = 0.92 mol of isopropyl alcohol

(0.92 mol of isopropyl alcohol/0.0297 kg of water) = 30.98 m

6. Convert 50% w/w glycerin to molarity (M). [NOTE—Glycerin has a MW of 92.1 g.]

From example 1, 50% w/w glycerin = 56.5% w/v glycerin

(56.5 g/100 mL) × (mol/92.1 g) × (1000 mL/L) = 6.13 M

7. Convert 50% w/w glycerin to % v/v. [NOTE—50% w/w of glycerin has a density of 1.13 g/mL; 100% glycerin has a density of 1.26 g/mL.]

50 g of glycerin/(1.26 g/mL) = 39.7 mL of glycerin

100 g of solution/(1.13 g/mL) = 88.5 mL of solution

(39.7 mL of glycerin/88.5 mL of solution) × 100% = 44.8% v/v

9. Convert 50% w/w glycerin to 1 in *R* ratio strength.

$1/R$ = (50 g of glycerin/100 g of solution)

$R = 2$

1 in R = 1 in 2

8.3.3 SOLID AND SEMISOLID IN SOLID AND SEMISOLID MIXTURE CONVERSIONS

The calculations used to convert from percent weight in weight (% w/w) to ppm and ratio strengths are illustrated as follows for fluocinonide and tolnaftate in topical semisolids and powders.

8.3.3.1 Calculating solid and semisolid in solid and semisolid mixture conversions

Examples—Solid and semisolid in solid and semisolid mixture conversions

1. Convert 0.05% w/w fluocinonide ointment to ppm.

(0.05 g/100 g) × (1×10^6 ppm) = 500 ppm

2. Convert 1.5% w/w tolnaftate powder to 1:*R* ratio strength.

$1/R$ = (1.5 g of tolnaftate/100 g of powder)

$R = 67$

$1:R = 1:67$

3. Convert 1% w/w tolnaftate in talcum powder to *X*:*Y* ratio strength.

100 g of powder – 1 g of tolnaftate = 99 g of talcum

$X:Y$ = 1 g of tolnaftate:99 g of talcum

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8.4 Dilution and Concentration

A more concentrated solution can be diluted to a lower concentration to obtain appropriate strength and precision when compounding preparations. Powders and semisolid mixtures can be triturated or mixed to achieve lower concentrations. The amount of an ingredient in the diluted mixture is the same as that in the portion of the more concentrated source used to make the dilution; thus, the following equation can be applied to dilution problems $(Q_1)(C_1) = (Q_2)(C_2)$, where Q_1 and Q_2 are the quantity of solutions 1 and 2, respectively, and C_1 and C_2 are concentrations of solutions 1 and 2, respectively. Any quantities and concentration terms may be used but the units of those terms must be the same on both sides of the equation.

8.4.1 CALCULATING DILUTION AND CONCENTRATION

Examples—Dilutions and fortifications

1. Semisolid dilution

Calculate the quantity (Q_2), in g, of diluent that must be added to 60 g of a 10% w/w ointment to make a 5% w/w ointment.

$$(Q_1) = 60 \text{ g}, (C_1) = 10\% \text{ w/w}, \text{ and } (C_2) = 5\% \text{ w/w}$$

$$60 \text{ g} \times 10\% \text{ w/w} = (Q_2) \times 5\% \text{ w/w}$$

$$(Q_2) = 120 \text{ g}$$

$$120 \text{ g} - 60 \text{ g} = 60 \text{ g of diluent to be added}$$

2. Solid dilution

Calculate the amount of diluent that should be added to 10 g of a trituration (1 in 100) to make a mixture that contains 1 mg of drug in each 10 g of the final mixture.

$$\text{Convert mg to g: 1 mg of drug} = 0.001 \text{ g of drug}$$

$$10 \text{ g of mixture should contain } 0.001 \text{ g of drug}$$

$$(Q_1) = 10 \text{ g}, (C_1) = (1 \text{ in } 100), \text{ and } (C_2) = (0.001 \text{ in } 10)$$

$$10 \text{ g} \times (1/100) = (Q_2) \times (0.001/10)$$

$$(Q_2) = 1000 \text{ g}$$

Because the final mixture of 1000 g contains 10 g of the trituration, 990 g (or 1000 g – 10 g) of diluent is required to prepare the mixture at a concentration of 0.001 g of drug in each 10 g.

3. Liquid dilution

Calculate the percentage strength (C_2) of a solution obtained by diluting 400 mL of a 5.0% w/v solution to 800 mL.

$$(Q_1) = 400 \text{ mL}, (C_1) = 5.0\% \text{ w/v}, \text{ and } (Q_2) = 800 \text{ mL}$$

$$400 \text{ mL} \times 5\% \text{ w/v} = 800 \text{ mL} \times (C_2)$$

$$(C_2) = 2.5\% \text{ w/v}$$

4. Liquid fortification

Calculate the additional amount, in g, of codeine phosphate that need to be added to 180 mL of a 12 mg/5 mL elixir of acetaminophen with codeine to have a final concentration of 30 mg/5 mL of codeine phosphate.

$$\text{Amount to add} = \text{Total amount required} - \text{Amount present}$$

$$\text{Total amount required: } (30 \text{ mg/5 mL}) \times 180 \text{ mL} = 1080 \text{ mg of codeine phosphate}$$

$$\text{Amount present} = (12 \text{ mg of codeine/5 mL}) \times 180 \text{ mL} = 432 \text{ mg of codeine phosphate}$$

$$\text{Amount to add: } 1080 \text{ mg} - 432 \text{ mg} = 648 \text{ mg of codeine phosphate}$$

Change to read:

9. ALCOHOL ▲AND ETHANOL▲ (USP 1-MAY-2019)

To achieve compliance with the statements in the *General Notices* about alcohol and the *USP* monograph for *Alcohol*, some conventions and special calculations are needed. See *General Notices 5.20.20.1 In Compounded Preparations, 8.30 Alcohol Content*, and *Labeling* ⟨7⟩, *Labels and Labeling for Products in Other Categories, Alcohol* for information. The *USP* monograph for

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Alcohol states that it contains 92.3%–93.8% by weight corresponding to 94.9%–96.0% by volume of alcohol (C_2H_5OH) at 15.56°. The percent concentration for alcohol is generally taken to be 95% v/v of alcohol (C_2H_5OH) in water.

In summary:

- When the word alcohol is written on a prescription order or in a formula, as for example "alcohol 10 mL" or "dissolve in 5 mL of alcohol", the compounder should use the Alcohol, USP [that is 95% alcohol (C_2H_5OH)].
- When the word alcohol is written with a percent, for example "alcohol 20%", this means 20% v/v of alcohol (C_2H_5OH). If this percent is on a label of a commercial product, it means the product contains 20% v/v alcohol (C_2H_5OH). If this is part of a compounding formula, it means the compounder must add the equivalent of 20% v/v alcohol (C_2H_5OH), which may require special calculations.
- Labels of products and compounded preparations are to include the content of alcohol (C_2H_5OH) in % v/v. For compounded preparations, this value must often be calculated based on the volume(s) of alcohol-containing ingredients added.

For calculations when preparing compounded drug preparations using Alcohol, USP, the first step is to determine the quantity, in mL, of alcohol needed, and the second step is to determine the % v/v of alcohol (C_2H_5OH) in the final preparation so that it can be properly labeled.

9.1 Calculating Alcohol

EXAMPLES—ALCOHOL

1. Determine the quantity of alcohol needed for the prescription (see *Table 3*):

Table 3

Clindamycin	1%
Alcohol	15%
Propylene glycol	5%
Purified water, a sufficient quantity to make	60 mL

A. In this prescription order, the alcohol 15% means the preparation contains 15% v/v of alcohol (C_2H_5OH).

B. Calculate the quantity of alcohol (C_2H_5OH) needed for 60 mL of preparation:

$$\blacktriangle\frac{\text{15 mL of alcohol}}{\text{100 mL of preparation}} = \frac{x\text{ mL of alcohol}}{\text{60 mL of preparation}}$$

x = 9 mL of alcohol ▲(USP 1-May-2019)

C. ▲Because alcohol or ethanol content is required in drug labeling, the content in this preparation would be labeled as either alcohol 15%, or ethanol 14.3%, where 14.3% results from (15 mL × 0.9545) in which 0.9545 is the mean fraction by volume of ethanol in Alcohol, USP.▲ (USP 1-May-2019)

D. Determine the % v/v alcohol content for labeling. Because labeling of alcohol is in % v/v of alcohol (C_2H_5OH), the alcohol content of this preparation would be labeled: Alcohol 15%.

2. Determine the alcohol content, in % v/v, for the prescription (see *Table 4*):

Table 4

Castor oil	40 mL
Acacia	As needed
Alcohol	15 mL
Cherry syrup	20 mL
Purified water, a sufficient quantity to make	100 mL

▲

A. In this formulation, alcohol 15 mL means the preparation contains 15 mL of Alcohol, USP.

B. In 100 mL of preparation, calculate the % v/v of ethanol: (15 mL × 0.9545) = 14.3 mL of ethanol, where 0.9545 is the mean fraction by volume of ethanol in Alcohol, USP.

C. Because alcohol or ethanol content is required in drug labeling, the content in this preparation would be labeled as either alcohol 15% or ethanol 14.3%.

▲ (USP 1-May-2019)

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Change to read:

10. ALLIGATION ALTERNATE AND ALGEBRA METHODS ▲FOR COMBINING MULTIPLE STRENGTHS OF THE SAME ACTIVE PHARMACEUTICAL INGREDIENT▲ (USP 1-MAY-2019)

10.1 Alligation Alternate

Alligation is a method of determining the proportions in which substances of different strengths are mixed to yield a desired strength or concentration. Once the proportion is found, the calculation may be performed to find the exact amounts of substances required.

Set up the problem as follows.

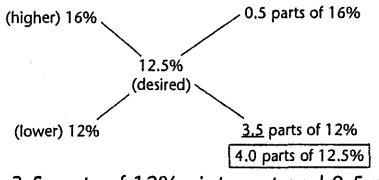
1. Place the desired percentage or concentration in the center.
2. Place the percentage of the substance with the lower strength on the lower left-hand side.
3. Place the percentage of the substance with the higher strength on the upper left-hand side.
4. Subtract the lower percentage from the desired percentage, and place the obtained difference on the upper right-hand side.
5. Subtract the desired percentage from the higher percentage, and place the obtained difference on the lower right-hand side.

The results on the right side determine how many parts of the two different percentage strengths should be mixed to produce the desired percentage strength of a drug mixture. The total parts will equal the final weight or volume of the preparation.

10.1.1 CALCULATING BY USING THE ALLIGATION ALTERNATE

Examples—Alligation alternate

1. Determine the amount of ointment containing 12% drug concentration and the amount of ointment containing 16% drug concentration must be used to make 1 kg of a preparation containing a 12.5% drug concentration.



In a total of 4 parts of 12.5% preparation, 3.5 parts of 12% ointment and 0.5 parts of 16% ointment are needed.

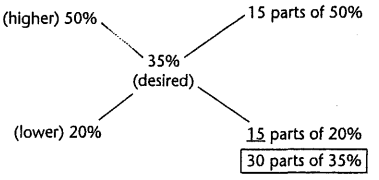
4 parts correspond to 1 kg or 1000 g.

1 part corresponds to 250 g.

3.5 parts correspond to 3.5 × 250 g or 875 g of 12% ointment.

0.5 parts correspond to 0.5 × 250 g or 125 g of 16% ointment.

2. Determine the volume, in mL, of 20% dextrose in water and 50% dextrose in water needed to make 750 mL of 35% dextrose in water.



In a total of 30 parts of 35% dextrose in water, 15 parts of 50% dextrose in water and 15 parts of 20% dextrose in water are required.

30 parts correspond to 750 mL.

15 parts correspond to 375 mL.

Thus, use 375 mL of the 20% solution and 375 mL of the 50% solution to prepare the preparation.

Change to read:

10.2 Algebra Method

The following algebraic equation may be used instead of alligation to solve problems of mixing two different strengths of the same ingredient:

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$(C_s \times Q_s) + (C_w \times Q_w) = (C_f \times Q_f)$, where C is concentration or strength, Q is the quantity; and the subscript s identifies the strongest strength, w identifies the weakest strength, f represents the final mixture with a strength less than s and greater than w, $(Q_s + Q_w) = Q_f$, $Q_s = (Q_f - Q_w)$, and $Q_w = (Q_f - Q_s)$.

10.2.1 CALCULATING BY USING THE ALGEBRA METHOD

Examples—Algebra method

1. Determine the amount, in g, of 16% w/w drug ointment and 12% w/w drug ointment required to prepare 1 kg of 12.5% w/w drug ointment.

$$(16\% \times Q_s) + [12\% \times (1000\ \text{g} - Q_s)] = 12.5\% \times 1000\ \text{g}$$

$$16\%\ Q_s + 120\ \text{g} - 12\%\ Q_s = 125\ \text{g}$$

$$4\%\ Q_s = 5\ \text{g}$$

$$Q_s = 5\ \text{g}/4\% = 125\ \text{g of 16\% ointment}$$

$$Q_w = 1000\ \text{g} - 125\ \text{g} = 875\ \text{g of 12\% ointment}$$

2. Determine the volume, in mL, of 10% dextrose injection and 50% dextrose injection needed to make 750 mL of 35% dextrose injection.

$$(50\% \times {}^{\blacktriangle}Q_{s\blacktriangle\,(\text{ERR 1-May-2019})}) + [10\% \times (750\ \text{mL} - {}^{\blacktriangle}Q_{s\blacktriangle\,(\text{ERR 1-May-2019})})] = 35\% \times 750\ \text{mL}$$

$$50\%\ {}^{\blacktriangle}Q_{s\blacktriangle\,(\text{ERR 1-May-2019})} + 75\ \text{mL} - 10\%\ {}^{\blacktriangle}Q_{s\blacktriangle\,(\text{ERR 1-May-2019})} = 262.5\ \text{mL}$$

$$40\%\ {}^{\blacktriangle}Q_{s\blacktriangle\,(\text{ERR 1-May-2019})} = 187.5\ \text{mL}$$

$${}^{\blacktriangle}Q_{s\blacktriangle\,(\text{ERR 1-May-2019})} = 187.5\ \text{mL}/40\% = 468.75\ \text{mL (470 mL practically)}$$

$${}^{\blacktriangle}Q_{w\blacktriangle\,(\text{ERR 1-May-2019})} = 750\ \text{mL} - 468.75\ \text{mL} = 281.25\ \text{mL (280 mL practically)}$$

Change to read:

11. ALIQUOT ▲DILUTIONS▲ (USP 1-MAY-2019)

When the quantity of drug desired requires a degree of precision in measurement that is beyond the capability of the available measuring devices, the pharmacist may use the aliquot method of measurement. It applies when potent drug substances are compounded, or when the total amount of the active drug in a single dose or individualized doses is less than the minimum accurately weighable quantity (MAWQ). Even if the amount of drug needed is greater than the MAWQ per unit, an aliquot will provide more material per unit, which will aid in handling and administration. Aliquot means "containing an exact number of times in something else;" the aliquot must be a proportional part of the total. Therefore, 5 is an aliquot part of 15, because 5 is contained exactly 3 times in 15. Both the total volume of solution or weight of powder triturate and the aliquot volume/weight should be easily and accurately measurable. If the solution or powder triturate is highly concentrated and a small error is made in measuring the aliquot, a large error can occur in the quantity of drug brought to the final formulation.

Aliquots can be: solid–solid, when the active drug and the diluents are solids; solid–liquids, when the active drug is solid and is to be incorporated into a liquid preparation, such as a solution, an emulsion, or a suspension; and liquid–liquid, when the active drug is liquid and the diluents are liquids. It can be a pure liquid or a concentrated solution of a drug. Aliquots of pure liquids are relatively uncommon because few drugs are liquid in their pure state. Aliquots involving concentrated solutions are more common.

There are two general methods to prepare aliquots:

1. Aliquot method 1 is applicable to drugs or substances that have to be within the degree of accuracy provided by the measuring device. It is the simplest method and can be applied to solid and liquid aliquots.
2. Aliquot method 2, also known as the dilution factor method, is useful when there is more flexibility in the amount of drug that may be measured.

   Aliquot Method 1

   A. The MAWQ amount of drug is measured.
   B. The drug is diluted with an arbitrary amount of diluent.
   C. The amount of dilution that will give the desired amount of drug is calculated, and the amount is measured.

   Aliquot Method 2

   A. The quantity of drug to be measured is determined by multiplying the amount of drug needed by an appropriately determined factor, called the dilution factor. The dilution factor must be a whole number more than or equal to the MAWQ divided by the amount of drug needed.
   B. An arbitrary amount of diluent is measured and added. The amount of diluent used can be determined by different methods, provided the amount of diluent chosen will give an aliquot greater than or equal to the MAWQ.

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C. The amount of aliquot needed is determined by multiplying the weight or volume of the dilution by the inverse of the dilution factor. Dilution factors are usually chosen to be whole numbers.

The general calculations can be shown as:

$$A/B = C/D$$

A = amount of drug desired
B = amount of drug measured
C = amount of drug in aliquot
D = aliquot total amount

11.1 Calculating Aliquots

EXAMPLES—ALIQUOTS

1. Solid-in-liquid dilution (Aliquot Method 1)
Prepare 100 mL of a solution containing 0.2 mg/mL of clonidine using water as the diluent. To prepare this solution, 20 mg of clonidine is needed.
   A. Select the weight of drug desired (A) to be equal to or greater than the MAWQ. In this situation, the MAWQ of the balance is 120 mg.
   B. Select the aliquot volume (D) in which the desired amount of drug (C) will be contained. This establishes the concentration of the solution to be prepared. Clonidine solubility is 1 g/13 mL, so if 5 mL is selected as the aliquot volume, the concentration in that solution will be 20 mg/5 mL. Therefore, solubility will not be a problem in this aqueous solution.
   C. Using the preceding formula, calculate the volume of solution (B) to be prepared.

$$120 \text{ mg of clonidine}/B = 20 \text{ mg of clonidine}/5 \text{ mL of aliquot}$$

$$B = 30 \text{ mL}$$

   D. Prepare the solution containing 120 mg of clonidine in 30 mL of *Purified Water*. Transfer a 5-mL aliquot from this solution to a final container, and add sufficient *Purified Water* to bring the formulation to a final volume of 100 mL.
2. Solid-in-solid dilution (Aliquot Method 2)
Prepare an individual dose of codeine phosphate 20 mg.
   A. Select a dilution factor that will yield a quantity that is greater than or equal to the MAWQ, and weigh this amount. In this case, the dilution factor may be greater than or equal to 6 because 6 × 20 mg = 120 mg. The smallest dilution factor that may be chosen is 6 if the MAWQ of the balance is 120 mg.
   B. Weigh an amount of diluent that will give an aliquot greater than or equal to the MAWQ. In this example, 600 mg of diluent is weighed.
   C. Mix the two powders thoroughly by geometric trituration in a mortar.
   D. Calculate the total weight of the dilution: 120 mg codeine phosphate + 600 mg diluent = 720 mg.
   E. Calculate the aliquot part of the dilution that contains 20 mg of codeine phosphate by multiplying the total weight of the dilution by the inverse of the dilution factor: 720 mg × (1/6) = 120 mg.
   F. Weigh this calculated amount of the dilution (120 mg) to get the desired 20 mg of codeine phosphate per dose.

Change to read:

12. POWDER VOLUME ▲DISPLACEMENT IN LIQUIDS▲ (USP 1-MAY-2019)

12.1 Displacement in Suspension

12.1.1 CALCULATING POWDER VOLUME

Examples—Powder volume

1. Powder displacement in suspension
The directions to reconstitute a 150-mL bottle of an amoxicillin for oral suspension of 250 mg/5 mL require 111 mL of *Purified Water*. The physician has requested that the product be reconstituted at a concentration of 500 mg/5 mL. Calculate the amount of *Purified Water* required for the higher concentration.
   A. Calculate the volume of the suspension occupied by the amoxicillin powder:

$$150 \text{ mL} - 111 \text{ mL} = 39 \text{ mL}$$

   B. Calculate the quantity of amoxicillin present in the entire bottle:

$$150 \text{ mL} \times (250 \text{ mg}/5 \text{ mL}) = 7500 \text{ mg}$$

   C. Calculate the total volume of the suspension at the requested concentration (500 mg/5 mL):

$$7500 \text{ mg}/(500 \text{ mg}/5 \text{ mL}) = 75 \text{ mL}$$

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D. Calculate the volume of *Purified Water* needed to reconstitute the powder by subtracting the powder volume calculated in step a:

75 mL – 39 mL = 36 mL of *Purified Water*

[NOTE—Such formulations may be too viscous to flow freely.]

2. Powder volume in drugs for injection
If the powder volume of 250 mg of ceftriaxone for injection is 0.1 mL, calculate the amount of diluent that should be added to 500 mg of ceftriaxone for injection to make a suspension with a concentration of 250 mg/mL.

A. Calculate the total volume of injection:

500 mg/(250 mg/mL) = 2 mL

B. Calculate the volume occupied by 500 mg of ceftriaxone for injection:

500 mg/(250 mg/0.1 mL) = 0.2 mL

C. Calculate the volume of the diluent required:

(2 mL of suspension) – (0.2 mL of ceftriaxone for injection) = 1.8 mL of diluent

13. INTRAVENOUS FLOW OR INFUSION RATES

Intravenous (IV) solutions and emulsions may be administered by gravity flow and infusion or syringe pumps. Gravity-flow IV sets are regulated by an adjustable clamp on the tubing, and the approximate flow rate is determined by counting the number of drops per 10–15 seconds, then adjusting that to a per minute rate. Manufactured IV sets are typically calibrated to deliver from 15 to 60 drops/mL, depending on the particular set.

13.1 Solving by Multiple or Separate Steps

As in previous sections, the following examples may be solved by multiple separate steps, or a single-DUA procedure.

13.1.1 CALCULATING INTRAVENOUS FLOW OR INFUSION RATES

Examples—IV or infusion rates

1. An IV infusion of dextrose 5% in water with 20 mEq of potassium chloride is to be administered to a 6-year-old child at the rate of 12 mL/hour. An IV administration set that delivers 60 drops/mL is available. Calculate the flow rate in drops per minute:

$$\frac{12\ \text{mL}}{\text{hour}} \times \frac{60\ \text{drops}}{\text{mL}} \times \frac{\text{hour}}{60\ \text{minutes}} = \frac{12\ \text{drops}}{\text{minute}}$$

2. A 63.6-kg patient is admitted to the Emergency Department and requires a dopamine hydrochloride infusion to maintain an adequate blood pressure. The drug is ordered at an initial rate of 2 mcg/kg/minute. A 400-mg/250-mL dopamine hydrochloride injection is available. Calculate the flow rate in mL/hour to be administered by infusion pump:

$$63.6\ \text{kg} \times \frac{2\ \mu\text{g}}{\text{kg} \cdot \text{minute}} \times \frac{250\ \text{mL}}{400\ \text{mg}} \times \frac{\text{mg}}{1000\ \mu\text{g}} \times \frac{60\ \text{minutes}}{\text{hour}} = \frac{4.8\ \text{mL}}{\text{hour}}$$

Change to read:

14. ▲OSMOLARITY AND TONICITY OF▲ (USP 1-MAY-2019) SOLUTIONS

The following discussion and calculations have therapeutic implications in preparations of dosage forms intended for ophthalmic, subcutaneous, intravenous, and intrathecal administration as well as for neonatal use.

14.1 Tonicity

Cells of the body, such as erythrocytes, will neither swell nor shrink when placed in a solution that is isotonic with body fluids. The measurement of tonicity, however, which is a physiological property, is somewhat difficult. A 0.9% w/v sodium chloride injection, which has a freezing point (FP) of –0.52°, is both isotonic and isoosmotic with body fluids. In contrast to isotonicity, FP depression is a physical property. Some solutions that are isoosmotic with body fluids are not isotonic, because they contain solutes to which cells are freely permeable rather than semipermeable. Freely permeable solutes (e.g., boric acid and urea) can cause erythrocyte lysis, i.e., behave as if they were hypotonic in concentrations that are hyperosmotic relative to body fluids. Nevertheless, many pharmaceutical products are prepared using FP data or related sodium chloride data to prepare solutions that are isoosmotic with body fluids. A closely related topic is osmolarity (see *Osmolality and Osmolarity* ⟨785⟩).

FP data or sodium chloride equivalents of pharmaceuticals and excipients (see *Table 5*) may be used to prepare isoosmotic solutions, as shown in the following examples.

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14.1.1 CALCULATING TONICITY

Example—Tonicity

Determine the amount of sodium chloride (NaCl) required to prepare 60 mL of an isoosmotic solution of atropine sulfate injection 0.5% using the E values and the FP depression values in *Table 5*.

Table 5. Sodium Chloride Equivalents (E) and FP Depressions for a 1% Solution of the Drug or Excipient

Drug or Excipient	E	FP Depression
Atropine sulfate	0.13	0.075
Sodium chloride	1.00	0.576

Using the E values:

1. The total amount of substances equivalent to a 0.9% sodium chloride injection = (0.9 g/100 mL) × 60 mL = 0.54 g.
2. The amount of atropine sulfate required = (0.5 g/100 mL) × 60 mL = 0.3 g.
3. 1 g of atropine sulfate is equivalent to 0.13 g of sodium chloride.
4. 0.3 g of atropine sulfate is equivalent to 0.3 × 0.13 g = 0.039 g of sodium chloride.
5. Thus, the required amount of sodium chloride is 0.54 g – 0.039 g = 0.501 g or 0.5 g.

Using FP depression values:

1. The FP depression required is 0.52°.
2. A 1% solution of atropine sulfate causes an FP depression of 0.075°.
3. A 0.5% solution of atropine sulfate causes an FP depression of 0.5 × 0.075° = 0.0375°.
4. The additional FP depression required is 0.52° – 0.0375° = 0.483°.
5. A 1% solution of sodium chloride causes an FP depression of 0.576°.
6. Therefore, an FP depression of 1° is caused by a 1%/0.576 = 1.736% solution of sodium chloride.
7. 1.736% × 0.483 = 0.838% solution of sodium chloride causes an FP depression of 0.482°.
8. The required amount of sodium chloride is (0.838%) × 60 mL = 0.502 g or 0.5 g.

Change to read:

15. pH AND ▲pH▲ (USP 1-MAY-2019) BUFFERS ▲▲ (USP 1-MAY-2019)

15.1 pH Calculations

See *Appendix 1* for logarithmic definitions and applications.

pH = –log $[H_3O^+]$, and pKa = –log $([H_3O^+][A^-])/[HA]$, where $[H_3O^+]$ is the hydrodium ion concentration in an aqueous solution, $[A^-]$ is the ionic form of the relevant acid, and Ka is the ionization constant of either a monoprotic acid or a particular proton from a polyprotic acid in aqueous solution. The $[H^+]$ = the antilogarithm of (–pH) or 10^{-pH}; and Ka = the antilogarithm of (–pKa) or 10^{-pKa}.

The pH of an aqueous solution containing a weak acid may be calculated using the Henderson–Hasselbalch equation:

$$pH = pKa + \log [(\text{base form})/(\text{acid form})]$$

The buffer equation symbol (↔) represents the equilibrium between conjugate base and acid forms or pairs of the same molecule. It is called the buffer equation, because small changes in the ratio of concentrations of the conjugate forms result in a logarithmically smaller change in pH. The salt form can be an acid or base, depending on structure; thus, its conjugate form is a base or acid, respectively.

Example 1:

B and BH^+ represent a nonionized or "free" base and cationic acid pair, $BH^+ \leftrightarrow B^+H^+$

Example 2:

HA and A^- represent a nonionized or "free" acid and anionic base pair, $HA \leftrightarrow A^- + H^+$

Example 3:

H_nA^- and $H_{n-1}A^{2-}$, such as $H_2PO_4^-$ and HPO_4^{2-}, represent an anionic acid and anionic base relative to each other; the pKa = 7.2 for $H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+$.

15.1.1 CALCULATING pH

Example—pH

A solution contains 0.020 mol/L of sodium acetate and 0.010 mol/L of acetic acid, which has a pKa value of 4.76. Calculate the pH and the $[H^+]$ of the solution as follows:

$$pH = 4.76 + \log (0.020/0.010) = 5.06$$

$$[H^+] = \text{antilogarithm of } (-5.06) = 8.69 \times 10^{-6}$$

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15.2 Buffer Solutions

15.2.1 DEFINITION

A buffer solution is an aqueous solution that resists a change in pH when small quantities of acid or base are added, when diluted with the solvent, or when the temperature changes. Most buffer solutions are mixtures of a weak acid and one of its salts, or mixtures of a weak base and one of its salts. Water and solutions of a neutral salt, such as sodium chloride, have very little ability to resist the change of pH and are not capable of effective buffer action.

15.2.2 PREPARATION, USE, AND STORAGE OF BUFFER SOLUTIONS

Buffer solutions for Pharmacopeial tests should be prepared using freshly boiled and cooled water (see *Reagents, Indicators and Solutions—Solutions, Buffer Solutions*). They should be stored in containers such as Type I glass bottles and used within 3 months of preparation.

Buffers used in physiological systems are carefully chosen so as to not interfere with the pharmacological activity of the medicament or the normal function of the organism. Commonly used buffers in parenteral products, for example, include: the nonionized acid and base salt pairs of acetic acid and sodium acetate, citric acid and sodium citrate, glutamic acid and sodium glutamate, and monopotassium or monosodium phosphate and dipotassium or disodium phosphate; and the acid salt and nonionized base pair tris(hydroxymethyl)aminomethane hydrochloride and tris(hydroxymethyl)aminomethane. Buffer solutions should be freshly prepared.

The Henderson–Hasselbalch equation, noted in *15.1 pH Calculations*, allows calculation of the pH and concentrations of conjugate pairs of weak acids and their salts and weak bases and their salts in buffer solutions when the pKa of the acid form of the buffer pair is known. Appropriately modified, this equation may be applied to buffer solutions composed of a weak base and its salt.

15.2.3 BUFFER CAPACITY

The buffer capacity of a solution is the measurement of the ability of that solution to resist a change in pH upon addition of small quantities of a strong acid or base. An aqueous solution has a buffer capacity of 1 when 1 L of the buffer solution requires 1 g equivalent of strong acid or base to change the pH by 1 unit. Therefore, the smaller the pH change upon the addition of a specified amount of acid or base, the greater the buffer capacity of the buffer solution. Usually, in analysis, much smaller volumes of buffer are used to determine the buffer capacity. An approximate formula for calculating the buffer capacity is g equivalents of strong acid or base added per L of buffer solution per unit of pH change, i.e., (g equivalents/L)/(pH change).

15.2.4 CALCULATING BUFFER CAPACITY

Example—Buffer capacity

The addition of 0.01 g equivalents of sodium hydroxide to 0.25 L of a buffer solution produced a pH change of 0.50. The buffer capacity of the buffer solution is calculated as follows:

$$(0.01\ \text{Eq}/0.25\ \text{L})/0.50\ \text{pH change} = 0.08(\text{Eq/L})/(\text{pH change})$$

Change to read:

16. TEMPERATURES

The relationship between Celsius or Centigrade (°C) and Fahrenheit (°F) temperature scale is expressed by the following equations:

$$°C = (°F - 32) \times (5/9)$$

$$°F = (°C \times 1.8) + 32$$

16.1 USP Temperatures

According to the *General Notices 8.180 Temperatures*, temperatures are expressed in centigrade (Celsius) degrees, and all other measurements are made at 25° unless otherwise indicated. For instructional purposes, °F is shown in the examples.

16.1.1 CALCULATING TEMPERATURES

Examples—Temperatures

1. Convert 77°F to Celsius degrees.

$$°C = (77°F - 32) \times (5/9) = 25°C$$

2. Convert 30°C to Fahrenheit degrees.

$$°F = (30°C \times 1.8) + 32 = 86°F$$

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The relationship between the Kelvin or absolute (K) and the Celsius (°C) scales is expressed by the equation:

$$K = °C + ▲273.15▲ \text{ (USP 1-May-2019)}$$

17. ENDOTOXINS

An endotoxin is a lipopolysaccharide that comes from a particular source, where species and strain number are usually indicated.

17.1 Endotoxin Concentrations

For more information concerning endotoxins, see *Bacterial Endotoxins Test* ⟨85⟩.

17.1.1 CALCULATING ENDOTOXINS

Example—Endotoxins

A 71.8-kg patient is to receive an intrathecal infusion of morphine sulfate at a rate of 0.3 mg/hour. The solution will be prepared by diluting preservative-free morphine sulfate injection, which contains 10 mg/mL of morphine sulfate, with 0.9% sodium chloride injection to produce an infusion rate of 2 mL/hour.

1. Determine the volume, in mL, of morphine sulfate injection (10 mg/mL) and 0.9% sodium chloride injection needed to prepare a 24-hour infusion.

0.3 mg of morphine sulfate per hour × 24 hours = 7.2 mg of morphine sulfate

7.2 mg of morphine sulfate/(10 mg/mL) = 0.72 mL of morphine sulfate injection

2 mL of infusion per hour × 24 h = 48 mL of total volume

48 mL total volume − 0.72 mL morphine sulfate injection = 47.28 mL of 0.9% sodium chloride injection

2. Calculate the maximum potential endotoxin load per hour for this preparation. [NOTE—*USP* monographs specify upper limits of 14.29 USP Endotoxin Units (EU)/mg of morphine sulfate in injections for intrathecal use, and 0.5 EU/mL for injections containing 0.5%–0.9% sodium chloride.]

7.2 mg of morphine sulfate injection × 14.29 EU/mg of morphine sulfate = 102.89 EU from morphine sulfate

47.28 mL of sodium chloride injection × 0.5 EU/mL = 23.64 EU from 0.9% sodium chloride injection

Endotoxin load = 102.89 EU + 23.64 EU = 126.53 EU

126.53 EU/24 hour = 5.27 EU/hour

3. Determine if the endotoxin load in step 2 exceeds the allowable USP limit for this patient. [NOTE—The maximum endotoxin load by intrathecal administration is 0.2 EU/kg/hour (see ⟨85⟩).]

Maximum endotoxin load = (0.2 EU/kg/hour) × 71.8 kg-patient = 14.36 EU/hour

The endotoxin load of 5.27 EU/hour does not exceed the allowable limit of 14.36 EU/hour.

Change to read:

18. STABILITY ▲KINETICS▲ (USP 1-MAY-2019) AND EXPIRATION DATE ▲PREDICTION▲ (USP 1-MAY-2019)

18.1 Stability Based on Rate Calculations

Calculation of a predetermined minimum percentage of initial drug strength or other quality parameter, e.g., in vitro dissolution of active pharmaceutical ingredients (APIs) or active drugs in solid oral dosage forms, is based on component-specific assays and other validated scientific testing. The expiration date or time elapsed until such minimum acceptable limits are reached for a specific manufactured product is exclusive to the specific formulation, packaging, and environmental conditions, e.g., temperature, humidity, and illumination, to which the item is subjected. See also ⟨659⟩, ⟨795⟩, ⟨797⟩, ⟨1163⟩,and ⟨1191⟩.

The degradation or concentration loss rates or kinetics of most APIs can be accurately represented or modeled by either zero order (constant) or first order (mono-exponential) rate equations. Zero order calculations are generally applicable to solids, semisolids, suspensions in which a majority of the drug strength is present as solid particles, and auto-oxidation in solutions. First order calculations are generally applicable for drug hydrolysis in solutions.

18.2 Zero Order Rate Calculations

The isothermal zero order or constant rate equation for a particular formulation is $C = C_0 - kt$, where C is the concentration of API at any time, C_0 is the concentration at origination or time zero, k is the reaction rate constant, and t is any time after

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origination or zero. The values and units of the rate, dC/dt, and rate constant, k, are the same for zero order processes, i.e., the units are concentration/time, such as mg/mL/day.

18.2.1 ZERO ORDER RATE EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate calculations of the zero order rate equation from original concentration assay and time data, and an expiration date using that equation.

18.2.1.1 Calculating zero order rate

Examples—Zero order rate

1. Calculate the zero order rate equation based on the assay results for a drug suspension at 25° (see *Table 6*):

Table 6

C (mg/mL)	t (days)
49	3
47.5	8
44.8	17
42.3	26

Linear regression of the C (ordinate) versus t (abscissa) values yields the equation, $C = 49.84 - 0.292t$ with a correlation coefficient of 0.9996.

2. Calculate the time when $C = 0.9 \times C_0$, i.e., the expiration date where the concentration will be 90% of the original concentration (t_{90}):

$$C = 49.84 - 0.292t$$

$$0.9 \times 49.84 = 49.84 - 0.292(t_{90})$$

$$t_{90} = (44.86 - 49.84)/-0.292 = 17.05 \text{ days}$$

3. Using the previous linear regression equation, calculate the C of the drug suspension at 25° when $t = 12$ days:

$$C = 49.84 - (0.292 \times 12) = 46.34 \text{ mg/mL}$$

4. Calculate t when $C = 45$ mg/mL:

$$45 = 49.84 - 0.292t$$

$$t = (45 - 49.84)/-0.292 = 16.6 \text{ days}$$

18.2.2 ZERO ORDER VALUES CALCULATED FROM A RATE EQUATION

The following are examples of expiration dates calculated from a rate equation derived from original concentration assay and time data.

18.2.2.1 Calculating zero order values from a rate equation

Examples—Zero order from a rate equation

1. Calculate the t_{80} expiration date of a drug cream at 25° using the equation, $C = 0.05 - 0.0003t$, where the C unit is % w/w and the t unit is months. At t_{80}, $C = 0.8C_0$.

$$0.8 \times 0.05 = 0.05 - 0.0003(t_{80})$$

$$t_{80} = 33.3 \text{ months}$$

2. Calculate the t_{80} expiration date of the drug cream formulation in example 1, but for which C_0 is 0.1:

$$0.8 \times 0.1 = 0.1 - 0.0003(t_{80})$$

$$t_{80} = 66.7 \text{ months}$$

18.3 First Order Rate Calculations

The isothermal first order rate equation for a particular formulation in exponential form is $C = C_0e^{-kt}$, and in linear form is $\ln(C) = \ln(C_0) - kt$, where C is the concentration of an API at any time, C_0 is the concentration at origination or time zero, k is the reaction rate constant, and t is any time after origination or zero. The constantly changing rate, dC/dt, and rate constant, k, are not the same for first order processes. The rate units are concentration/time, e.g., mg/mL/hour, but the rate constant unit is reciprocal time, time^{-1}, e.g., hour^{-1}.

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18.3.1 FIRST ORDER LINEAR RATE EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate calculation of the linear first order rate equation from original concentration assay and time data and calculation of an expiration date using that equation.

18.3.1.1 Calculating first order linear rate equations

Example—First order linear rate

1. Calculate the linear first order rate equation based on the assay results for a drug solution at 27° (see *Table 7*):

Table 7

C (mg/mL)	t (h)
12.3	2
11.9	6
11.5	14
10.6	24

Linear regression of the ln(C) (ordinate) versus t (abscissa) values yields the equation, $\ln(C) = 2.522 - 0.0065t$ with a correlation coefficient of 0.992.

2. From the linear regression equation, calculate the time when 95% of the original concentration is reached, t_{95}, when $C = 0.95C_0$, which is the predetermined expiration date:

$$\ln(C_0) = 2.522;\ \text{thus},\ C_0 = e^{2.522} = 12.45\ \text{mg/mL}$$

$$\ln(0.95 \times 12.45) = 2.522 - 0.0065(t_{95})$$

$$t_{95} = (2.470 - 2.522)/-0.0065 = 8\ \text{hours}$$

18.3.2 FIRST ORDER VALUES CALCULATED FROM A LINEAR EQUATION

The following are examples of an expiration date, concentration, and time calculated for the same drug solution at 22° from the rate equation, $\ln(C) = 4.382 - 0.076t$, where the C units are mcg/mL and the t unit is days, derived from the original concentration assay and time data.

18.3.2.1 Calculating first order values from a linear rate equation

Examples—First order from a linear rate

1. Calculate the t_{90} expiration date:

$$\ln(C_0) = 4.382;\ \text{thus},\ C_0 = e^{4.382} = 80$$

$$\ln(0.9 \times 80) = 4.382 - 0.076(t_{90})$$

$$t_{90} = (4.277 - 4.382)/-0.076 = 1.4\ \text{days}$$

2. Calculate the time at which $C = 75$ mcg/mL:

$$\ln(75) = 4.382 - 0.076t$$

$$t = (4.317 - 4.382)/-0.076 = 0.86\ \text{day}$$

3. Calculate whether $C = 70$ mcg/mL occurs before or after t_{90}:

$$\ln(70) = 4.382 - 0.076t$$

$$t = (4.248 - 4.382)/-0.076 = 1.8\ \text{days}$$

$C = 70$ mcg/mL occurs at 1.8 days, after a t_{90} of 1.4 days

18.3.3 FIRST ORDER EXPIRATION DATE CALCULATED FROM TWO VALUES OF CONCENTRATION AND TIME

When degradation or other cause of concentration loss is known from experience or reference information to obey first order kinetics, the rate constant can be accurately estimated from accurate assays of only two concentrations at their respective times. In this case, the linear first order rate equation, $\ln(C) = \ln(C_0) - kt$, may be transformed or integrated as $\ln(C_2) = \ln(C_1) - k(t_2 - t_1)$, which when rearranged is $k = \ln(C_1/C_2)/(t_2 - t_1)$. The following examples apply these equations to calculate expiration dates, concentrations, and times.

18.3.3.1 Calculating first order expiration date from two values

Examples—First order expiration date from two values

1. At 25°, the concentration of an antibiotic in solution was 89 mg/mL after 3 hours and 74 mg/mL after 8 hours. Calculate the initial concentration at time zero:

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$$k = \ln(89/74)/(8 - 3) = 0.037 \text{ hour}^{-1}$$

$$\ln(89) = \ln(C_0) - (0.037 \text{ hour}^{-1} \times 3 \text{ hour})$$

$$\ln(C_0) = 4.489 + 0.111 = 4.6$$

$$C_0 = e^{4.6} = 99.5 \text{ mg/mL}$$

2. Calculate the t_{90} expiration date using the data in example 1. At t_{90}, $C = 0.9C_0$.

$$\ln(0.9 \times 99.5) = \ln(99.5) - 0.037(t_{90})$$

$$t_{90} = (4.495 - 4.600)/-0.037 \text{ hour}^{-1} = 2.8 \text{ hours}$$

3. Calculate the concentration at 6 hours using the data in example 1:

$$\ln(C) = \ln(99.5) - (0.037 \times 6)$$

$$\ln(C) = 4.378$$

$$C = e^{4.378} = 79.7 \text{ mg/mL}$$

18.3.4 FIRST ORDER TIMES, t_n, FOR 0.n FRACTION OR n% OF REMAINING ORIGINAL CONCENTRATION

The two most common first order pharmaceutical t_n values are the t_{50}, which is a primary parameter factor in clinical pharmacokinetics, and the t_{90}, which is the most common stability shelf life or expiration date. Values of any t_n, where $0 < n < 100$, are derived from the linear first order equation, $\ln(C) = \ln(C_0) - kt$. The equations for t_{50} and t_{90} in particular are derived in the following examples. The value of k by definition is constant for a specific drug chemical in a specific formulation at a specific temperature; thus, t_n values derived from such values of k are also constant.

18.3.4.1 Calculating first order times for remaining original concentrations

Examples—First order times for remaining original concentrations

1. At t_n, $C = 0.n \times C_0$.

$$\ln(0.n \times C_0) = \ln(C_0) - kt_n$$

$$t_n = [\ln(0.n \times C_0) - \ln(C_0)]/-k = \ln[(0.n \times C_0)/C_0]/-k = \ln(0.n)/-k$$

$$t_n = \ln(0.n)/-k$$

2. At t_{50}, $C = 0.5(C_0)$.

$$\ln(0.5 \times C_0) = \ln(C_0) - kt_{50}$$

$$t_{50} = [\ln(0.5 \times C_0) - \ln(C_0)]/-k = \ln[(0.5 \times C_0)/C_0]/-k = \ln(0.5)/-k = -0.693/-k = 0.693/k$$

$$t_{50} = 0.693/k$$

3. At t_{90}, $C = 0.9(C_0)$.

$$\ln(0.9 \times C_0) = \ln(C_0) - kt_{90}$$

$$t_{90} = [\ln(0.9 \times C_0) - \ln(C_0)]/-k = \ln[(0.9 \times C_0)/C_0]/-k = \ln(0.9)/-k = -0.105/-k = 0.105/k$$

$$t_{90} = 0.105/k$$

18.4 Stability Prediction Based on Arrhenius Theory

The basis of the Arrhenius theory is that reaction rates and rate constants change exponentially in the direction of arithmetic temperature change. The pharmaceutical application of the Arrhenius theory is based on scientifically accurate and statistically valid assay data obtained at three or more temperatures that are ≥10° warmer than the intended drug storage temperature and each other. The Arrhenius equation may be expressed in an exponential form, $k = Ae^{-(Ea/RT)}$, a linear form, $\ln(k) = \ln(A) - (E_a/RT)$, and an integrated form, $\ln(k_2/k_1) = E_a(T_2 - T_1)/[R(T_2 \times T_1)]$, where k, k_1, and k_2 are isothermal rate constants, A is a thermodynamic factor, E_a is energy of activation for the degradation reaction, R is the gas constant (1.987×10^{-3} kcal mol^{-1}K^{-1} or 8.314×10^{-3} J K^{-1} mol^{-1}), and T, T_1, and T_2 are absolute or Kelvin temperatures.

18.4.1 ARRHENIUS LINEAR EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate derivation of a linear Arrhenius equation from original assay data and its application to predicting a drug stability expiration date at a cooler or lower storage temperature.

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18.4.1.1 Calculating Arrhenius equations

Examples—Arrhenius equations

1. Calculate the linear Arrhenius equation based on the rate constants and temperatures for a beta-lactam antibiotic that decomposes in solution at a first order rate (see *Table 8*):

Table 8

T (°C)	T (K)	$1/T$ (K^{-1})	k ($hour^{-1}$)	ln(k)
40	313	3.195×10^{-3}	0.0014	−6.571
50	323	3.096×10^{-3}	0.005	−5.298
60	333	3.003×10^{-3}	0.016	−4.135

Linear regression of the ln(k) (ordinate) *versus* $1/T$ (abscissa) values yields the equation, $\ln(k) = 33.977 - (12{,}689/T)$ with a correlation coefficient of 0.99997.

2. Calculate the t_{90} shelf life expiration date, in days, at 25°C (298 K) using the equation in example 1:

$$\ln(k_{25}) = 33.977 - (12{,}689/298) = 33.977 - 42.581 = -8.604$$

$$k_{25} = e^{-8.604} = 1.834 \times 10^{-4}\ hour^{-1} = 4.402 \times 10^{-3}\ day^{-1}$$

$$t_{90} = 0.105/k = 0.105/4.402 \times 10^{-3}\ day^{-1} = 23.85\ days$$

$$t_{90}\ \text{at } 25°C = 23.85\ days$$

18.4.2 STABILITY PREDICTIONS USING THE INTEGRATED ARRHENIUS EQUATION

The following examples illustrate stability predictions based on one accurately determined isothermal rate constant and adherence to the same degradation rate order, e.g., first order, at temperatures at which stability is to be calculated from the equation, $\ln(k_2/k_1) = E_a(T_2 - T_1)/[R(T_2 \times T_1)]$.

18.4.2.1 Calculating stability prediction using integrated Arrhenius equation

Example—Stability using integrated Arrhenius equations

1. Calculate the t_{85} stability expiration date at 4°C (277 K) for an ester hydrolysis with an E_a = 15 kcal/mol and k = 0.0045 $hour^{-1}$ at 23°C (296 K):

$$\ln(k_{277}/0.0045) = 15(277 - 296)/[1.987 \times 10^{-3}\ (277 \times 296)]$$

$$\ln(k_{277}) - \ln(0.0045) = -285/162.92$$

$$\ln(k_{277}) = -1.749 + \ln(0.0045) = -7.153$$

$$k_{277} = e^{-7.153} = 7.825 \times 10^{-4}\ hour^{-1}$$

$$t_{85} = \ln(0.850)/k_{277} = -0.163/-7.825 \times 10^{-4}\ hour^{-1} = 208.3\ hours$$

$$t_{85}\ \text{at } 4°C = 208.3\ hours\ (t_{85}\ \text{at } 23°C \text{ is } 36.2\ hours)$$

18.4.3 ARRHENIUS-BASED Q_{10} STABILITY ESTIMATION

The temperature coefficient (Q_{10}) represents the multiplicative factor by which a chemical reaction rate constant changes in the same direction as the temperature for each 10°C change. For drug molecules, Q_{10} ranges from 2 to 5, corresponding to an E_a range of 10–25 kcal/mol or 42–105 kJ/mol. A Q_{10} of 3 yields reasonable estimates of drug stability in the equation,

$$\left(t_n \text{ at } T_2\right) = \left(t_n \text{ at } T_1\right) / \left\{Q_{10}^{\left[\left(T_2 - T_1\right)/10\right]}\right\}$$

n = percentage of remaining C_0
T_1 = temperature at which t_n is known
T_2 = temperature at which t_n is to be estimated

Calculations using Q_{10} values of both 2 and 4 may be used to obtain the shortest or most conservative stability estimate, but Q_{10} = 3 is applied in the following two examples.

18.4.3.1 Calculating Arrhenius-based Q_{10} stability estimation

Example—Arrhenius-based Q_{10} stability

1. Estimate the t_{90} expiration date in hours of an antibiotic suspension stored in a closed automobile at 57° for which the 8° refrigeration t_{90} is 14 days.

$$t_{90}\ \text{at } 57° = [14\ days \times (24\ hours/day)]/\{3^{[(57-8)/10]}\} = 336\ hours/3^{4.9} = 336\ hours/217.7 = 1.54\ hours$$

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t_{90} at 57° = 1.54 hours

Add the following:

▲19. MEAN KINETIC TEMPERATURE

19.1 Definition of Terms

Mean kinetic temperature (MKT) is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT may be considered as an isothermal storage temperature that simulates the non-isothermal effects of storage temperature variation. It is not a simple arithmetic mean (see *Good Storage and Distribution Practices for Drug Products* ⟨1079⟩).

Controlled room temperature (CRT) is the temperature thermostatically maintained that encompasses the usual and customary working environment of 20°–25° (68°–77°F). The following conditions apply to the application of MKT to CRT:

- MKT must not exceed 25°.
- Excursions between 15° and 30° (59° and 86°F) that are experienced in pharmacies, hospitals, and warehouses, and during shipping are allowed.
- Provided the MKT does not exceed 25°, transient spikes up to 40° are permitted as long as they do not exceed 24 h. Spikes above 40° may be permitted only if the manufacturer provides documentation that such spikes are permitted.
- Articles may be labeled for storage at "controlled room temperature" or at "up to 25°," or other wording based on the same MKT (see ⟨659⟩).

19.2 MKT Equation

(See ⟨1079⟩.)

$$\text{MKT or } T_k = \frac{-\Delta H / R}{ln\left(\frac{\Sigma e^{-\Delta H/RT_1} + \cdots + e^{-\Delta H/RT_n}}{n}\right)}$$

ΔH =	83.144 kJ/mol[a]
R =	8.3144 × 10^{-3} kJ/mol · K (universal gas constant)
T_1 =	value for the temperature recorded during the first time period, e.g., the first week
T_n =	▲value for the temperature recorded during the *n*th time period, e.g., *n*th week▲ (ERR 1-May-2019)
n =	total number of storage temperatures recorded during the observation period

[a] ΔH = 83.144 kJ/mol or 19.86 kcal/mol is a typical or approximately average Arrhenius energy of activation, Ea, for hydrolysis of amide, ester, lactam and peptide bonds from more than 100 drug stability reports.[1,2,3]

[NOTE—All temperatures, T, are absolute temperatures in degrees Kelvin (K).]

19.3 MKT Equation Stepwise Solution

1. Calculate the inverse ln of the negative value of ($\Delta H/RT$) for each of the total n mean temperatures.
   For example, for 27°C or 300.15 K:

$$e^{-\Delta H/RT} = e^{-[(83.144\ \text{kJ/mol})/((8.3144 \times 10^{-3}\ \text{kJ/mol}\cdot\text{K}) \times 300.15\ \text{K})]} = e^{-(83.144/2.496)} = e^{-33.311}$$
$$= 3.414 \times 10^{-15}$$

2. Sum, Σ, all the step 1 values.
3. Divide the step 2 sum by n.
4. Take ln of the step 3 result.
5. Divide $-\Delta H/R$ (equivalent to −10,000 K) by the step 4 answer.

$$-\Delta H / R = (83.144\ \text{kJ/mol}) / (8.3144 \times 10^{-3}\ \text{kJ/mol}\cdot\text{K}) = -10{,}000\ \text{K}$$

6. MKT = step 5 answer.
7. Compare step 6 answer with MKT ≤25°C for determination of whether the temperature falls within the range for CRT.

[1] Kennon L. Use of models in determining chemical pharmaceutical stability. *J Pharm Sci.* 1964;53(7):815–8.
[2] Bailey LC, Medwick T. Mean kinetic temperature—a concept for storage of pharmaceuticals. *Pharm Forum.* 1993;19:6163–6.
[3] Yoshioka S, Stella VJ. *Stability of Drugs and Dosage Forms.* New York, NY: Kluwer Academic Publishers; 2002:62–5.

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19.4 Example Calculations of MKT for CRT Storage Evaluation

EXAMPLE 1—CALCULATION OF 1-MONTH MKT

1. Calculate the 1-month MKT in °C and determine whether it falls within the definition of CRT from the data in *Table 9* in which the far right-hand column comprises the step (1) calculation in *19.3 MKT Equation Stepwise Solution*.

Table 9

Week	Temperatures, *T*				$\Delta H/RT$	$e^{-\Delta H/RT}$
	Lowest (°C)[a]	Highest (°C)[a]	Mean (°C)[a]	Mean (K)		
1	24	28	26	299.15	33.428	3.037×10^{-15}
2	23	26	25	298.15	33.540	2.715×10^{-15}
3	19	24	22	295.15	33.881	1.930×10^{-15}
4	21	26	24	297.15	33.653	2.425×10^{-15}

[a] Rounded to nearest whole °C.

2. $\Sigma(3.037 \times 10^{-15} + 2.715 \times 10^{-15} + 1.930 \times 10^{-15} + 2.425 \times 10^{-15}) = 1.011 \times 10^{-14}$
3. $(1.011 \times 10^{-14})/4 = 2.528 \times 10^{-15}$
4. $\ln(2.528 \times 10^{-15}) = -33.612$
5. $-10{,}000\ \text{K}/-33.612 = 297.51\ \text{K}$
6. MKT (°C) = 297.51 K − 273.15 K = 24.36°C
7. MKT of 24.36°C meets the CRT between 20° and 25°C; thus, the MKT falls within the CRT range in ⟨659⟩.

EXAMPLE 2—CALCULATION OF ANNUAL MKT

1. Calculate the annual MKT in °C and determine whether the MKT falls within the range of CRT from the data in *Table 10* in which the far right-hand column comprises the step (1) calculation in *19.3 MKT Equation Stepwise Solution*.

Table 10

Month	Temperatures, *T*				$\Delta H/RT$	$e^{-\Delta H/RT}$
	Lowest (°C)[a]	Highest (°C)[a]	Mean (°C)[a]	Mean (K)		
April	23	27	25	298.15	33.540	2.715×10^{-15}
May	23	28	26	299.15	33.428	3.037×10^{-15}
June	22	25	24	297.15	33.653	2.425×10^{-15}
July	23	27	25	298.15	33.540	2.715×10^{-15}
August	27	29	28	301.15	33.206	3.791×10^{-15}
September	24	26	25	298.15	33.540	2.715×10^{-15}
October	24	27	26	299.15	33.428	3.037×10^{-15}
November	23	26	25	298.15	33.540	2.715×10^{-15}
December	23	27	25	298.15	33.540	2.715×10^{-15}
January	24	28	26	299.15	33.428	3.037×10^{-15}
February	21	24	23	296.15	33.767	2.164×10^{-15}
March	23	26	25	298.15	33.540	2.715×10^{-15}

[a] Rounded to nearest whole °C.

2. $\Sigma(2.715 \times 10^{-15} + 3.037 \times 10^{-15} + 2.425 \times 10^{-15} + 2.715 \times 10^{-15} + 3.791 \times 10^{-15} + 2.715 \times 10^{-15} + 3.037 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 3.037 \times 10^{-15} + 2.164 \times 10^{-15} + 2.715 \times 10^{-15}) = 3.378 \times 10^{-14}$
3. $3.378 \times 10^{-14}/12 = 2.815 \times 10^{-15}$
4. $\ln(2.815 \times 10^{-15}) = -33.504$
5. $-10{,}000\ \text{K}/-33.504 = 298.473\ \text{K}$
6. MKT (°C) = 298.473 K − 273.15 K = 25.32°C
7. 25.32°C exceeds the CRT limit of ≤ 25°C for MKT; thus, MKT does not fall within the CRT range in ⟨659⟩

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EXAMPLE 3—CALCULATION OF ANNUAL MKT

1. Calculate the annual MKT in °C and determine whether the MKT falls within the range of CRT from the data in *Table 11* in which the far right hand column comprises step (1) calculation in *19.4 Example Calculations of MKT for CRT Storage Evaluation.*

Table 11

Month	Temperature, *T*				$\Delta H/RT$	$e^{-\Delta H/RT}$
	Lowest, °C[a]	Highest, °C[a]	Mean, °C[a]	Mean, K[b]		
1	23	27	25	298.15	33.540	2.715×10^{-15}
2	22	28	25	298.15	33.540	2.715×10^{-15}
3	18	22	20	293.15	34.112	1.532×10^{-15}
4	23	27	25	298.15	33.540	2.715×10^{-15}
5	23	26	25	298.15	33.540	2.715×10^{-15}
6	24	26	25	298.15	33.540	2.715×10^{-15}
7	24	26	25	298.15	33.540	2.715×10^{-15}
8	27	33	30	303.15	32.987	4.720×10^{-15}
9	23	27	25	298.15	33.540	2.715×10^{-15}
10	24	25	25	298.15	33.540	2.715×10^{-15}
11	21	29	25	298.15	33.540	2.715×10^{-15}
12	23	26	25	298.15	33.540	2.715×10^{-15}

[a] Rounded to nearest whole °C.
[b] K = °C + 273.15

2. $\Sigma(2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 1.532 + \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 4.720 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} = 3.340 \times 10^{-14}$
3. $3.354 \times 10^{-14}/12 = 2.783 \times 10^{-15}$
4. $\ln(2.795 \times 10^{-15}) = -33.515$
5. $-10{,}000\text{K}/-33.511 = 298.372\text{K}$
6. MKT(°C) = 298.410 K − 273.15 K = 25.22°C
7. 25.22°C exceeds the CRT limit of ≤25°C for MKT; thus, MKT does not fall within the CRT range in ⟨659⟩

The 2 months of mean temperatures 20°C and 30°C, which arithmetically yield 2 months of mean 25°C, resulted in an MKT of 25.22°C, exceeding the range for CRT. This result confirms the statement, "...higher temperatures are given greater weight in the calculation..." in ⟨1079⟩. However, higher temperatures are not intentionally "given greater weight in the [MKT] calculation," but higher temperatures have a greater influence over the MKT result, because of the logarithmic or exponential increase in reaction rate with arithmetic or linear increase in temperature. ▲(USP 1-May-2019)

Change to read:

APPENDIX 1: ▲COMMON AND NATURAL▲ (USP 1-MAY-2019) LOGARITHMS

The logarithm of a number is the exponent or power to which a given base number must be raised to equal that number. Thus, the logarithm of Y to the base, b, equals X, or $\log_b(Y) = X$. The logarithm of 0 and all negative numbers is undefined or nonexistent. The logarithm of 1 is 0 and of numbers <1 is negative in all systems (see ▲*Table A-1*).▲ (USP 1-May-2019)

Table ▲A-1▲ (USP 1-May-2019) Common (or Briggsian) and Natural (or Napierian) Logarithms

Logarithmic System	Abbreviation or Symbol	Base Number	Format	Antilogarithm or Inverse Logarithm
Common	Log	10	$\log Y = X$	$10^X = Y$
Natural	Ln	e or 2.7183[a]	$\ln Y = X$	$e^X = Y$

[a] e is an irrational number derived from an infinite series of reciprocal whole number factorials, $e = 1 + 1/1! + 1/2! + 1/3! + 1/4! \ldots + 1/n!$, where n = infinity. e rounds to 2.7183 when $n \geq 8$.

The relationships between common and natural logarithms are the following:

1. $\log Y = \ln Y/\ln 10 = \ln Y/2.303$
2. $\ln Y = \ln 10 \times \log Y = 2.303 \times \log Y$

Rules for some common calculations with logarithms are shown in ▲*Table A-2*.▲ (USP 1-May-2019)

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Table ▲A-2▲ (USP 1-May-2019) Rules for Calculating with Logarithms

	Formula	Example
Additions and multiplications	$\ln(A) + \ln(B) = \ln(A \times B)$ $\log(A) + \log(B) = \log(A \times B)$	$\ln(0.62) + \ln(1.73) = \ln(0.62 \times 1.73) = \ln(1.0726) = 0.070$ $\log(5.7) + \log(0.43) = \log(5.7 \times 0.43) = \log(2.451) = 0.389$
Subtraction and quotients	$\ln(A) - \ln(B) = \ln(A/B)$ $\log(A) - \log(B) = \log(A/B)$	$\ln(0.5) - \ln(4) = \ln(0.5/4) = \ln(0.125) = -2.079$ $\log(1.57) - \log(2.48) = \log(1.57/2.48) = \log(0.6330645) = -0.199$
Simple non-base exponentials	$\ln(Y^Z) = Z \times \ln(Y)$ $\log(Y^Z) = Z \times \log(Y)$	$13.6^{-Z} = 1.25$ $\ln(1.25) = -Z \times \ln(13.6)$ $Z = -\ln(1.25)/\ln(13.6) = -0.223/2.610 = -0.085$ $0.57^Z = 2.3$ $\log(2.3) = Z \times \log(0.57)$ $Z = \log(2.3)/\log(0.57) = 0.362/-0.244 = -1.484$
Base exponentials	$\ln(a \times e^{\pm b}) = \ln(x) \leftrightarrow \ln(a) \pm b = \ln(x)$ $\log(a \times 10^{\pm b}) = \log(x) \leftrightarrow \log(a) \pm b = \log(x)$	$67 \times 10^b = 15.1$ $\log(67) + b = \log(15.1)$ $1.826 + b = 1.179$ $b = 1.179 - 1.826 = -0.647$

⟨1163⟩ QUALITY ASSURANCE IN PHARMACEUTICAL COMPOUNDING

Change to read:

INTRODUCTION

The need for a quality assurance system is well documented in *United States Pharmacopeia (USP)* chapters for compounded preparations (see *Quality Control* under *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and ▲*Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, *18. Quality Assurance and Quality Control*▲ (CN 1-May-2020)). A quality assurance program is guided by written procedures that define responsibilities and practices that ensure compounded preparations are produced with quality attributes appropriate to meet the needs of patients and health care professionals. The authority and responsibility for the Quality Assurance program should be clearly defined and implemented and should include at least the following nine separate but integrated components: (1) training; (2) standard operating procedures (SOPs); (3) documentation; (4) verification; (5) testing; (6) cleaning, disinfecting, and safety; (7) containers, packaging, repackaging, labeling, and storage; (8) outsourcing, if used; and (9) responsible personnel.

The definition of compounding for the purpose of this chapter is defined in general test chapter ⟨795⟩.

The safety, quality, and efficacy and/or benefit of compounded preparations depend on correct ingredients and calculations; accurate and precise measurements; appropriate formulation, facilities, equipment, and procedures; and prudent pharmaceutical judgment. As a final check, the compounder shall review each procedure in the compounding process. To ensure accuracy and completeness, the compounder shall observe the finished preparation to ensure that it appears as expected and shall investigate any discrepancies and take appropriate corrective action before the prescription is dispensed to the patient.

The water used in all aspects of compounding should meet the requirements of *Waters for Pharmaceutical Purposes* ⟨1231⟩.

Radiopharmaceuticals and radiolabeled materials have unique characteristics requiring additional quality assurances described in *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses* ⟨823⟩ and the ▲*Radiopharmaceuticals—Preparation, Compounding, Dispensing, and Repackaging* ⟨825⟩▲ (CN 1-May-2020).

The responsibilities of the compounder and compounding personnel can be found in chapters ⟨795⟩ and ⟨797⟩.

TRAINING

Personnel involved in nonsterile or sterile compounding require additional, specific training and periodic retraining beyond the training needed for routine dispensing duties. A thorough quality assurance program for compounded preparations requires documentation of both training and skill competency. In addition, the authority and responsibility for the QA program should be clearly defined as implemented. Training for nonsterile compounders should meet or exceed the standards set forth in ⟨795⟩, and personnel training for sterile preparation compounders should meet or exceed the standards set forth in ⟨797⟩.

STANDARD OPERATING PROCEDURES

SOPs for pharmaceutical compounding are documents that describe how to perform routine and expected tasks in the compounding environment, including but not limited to procedures involving:

- Beyond-Use dating
- Chemical and physical stability
- Cleaning and disinfecting
- Component quality evaluation
- Compounding methods

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- Dispensing
- Documentation
- Environmental quality and maintenance
- Equipment maintenance, calibration, and operation
- Formulation development
- Labeling
- Materials and final compounded preparation handling and storage
- Measuring and weighing
- Packaging and repackaging
- Patient monitoring, complaints, and adverse event reporting
- Patient or caregiver education and training
- Personnel cleanliness and garb
- Purchasing
- Quality Assurance and Continuous Quality Monitoring
- Safety
- Shipping
- Testing
- Training and retraining

SOPs are itemized instructions that describe when a task will be performed, how a task will be performed, who will perform the task, why the task is necessary, any limitations in performing the task, and what action to take when unacceptable deviations or discrepancies occur.

SOPs must be reviewed regularly and updated as necessary. Auditing and verifying compliance with established SOPs should be performed periodically. The SOP should be specific to each device and process used in compounding. Properly maintained and implemented SOPs are vital to preparation quality.

DOCUMENTATION

The purpose of documentation is to provide a record of all aspects of compounding operations and procedures that are described in this chapter, in ⟨795⟩, and in ⟨797⟩. Information on the compounding record should ideally be entered as the tasks are performed or as testing data is received. Compounding records should be reviewed for accuracy, completeness (as appropriate) and approved by QA personnel, prior to dispensing. Additionally, beyond-use dating and sterility studies, where appropriate, should be documented by reference to at least one of the following:

- Stability studies published in peer-reviewed literature,
- In-house or laboratory conducted stability and/or sterility studies,
- National compendia, or
- An extrapolation of above based on professional judgment.

Change to read:

VERIFICATION

Verification involves authoritatively signed assurance and documentation that a process, procedure, or piece of equipment is functioning properly and producing the expected results. The act of verification of a compounding procedure involves checking to ensure the calculations, weighing and measuring, order of mixing, and compounding techniques and equipment were appropriate and accurately performed. The quality of ingredients should be verified upon receipt (e.g., Certificate of Analysis, manufacturer's label on commmercial products, etc.). Verification may require outside laboratory testing when in-house capabilities are not adequate. Equipment verification methods are sometimes available from manufacturers of the specific equipment or can be developed in-house. The responsibility for assuring that equipment performance is verified, including work completed by contractors, resides with the compounder.

See ▲*Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Equipment and Components*.▲ (CN 1-May-2020)

TESTING

A quality assurance program for compounded preparations should include testing during the compounding process and of the finished compounded preparation, when appropriate, as described in chapters ⟨795⟩ and ⟨797⟩. The compounder should have a basic understanding of pharmaceutical analysis to ensure that valid results are obtained when tests are being conducted, whether they are done in-house or outsourced. Acceptance criteria shall be determined prior to testing. Testing every compounded preparation is neither practical nor officially required, but compounders should conduct visual inspections and know: (1) the importance of testing in the overall quality program in the compounding facility, (2) when to test, (3) what to test, (4) what appropriate method(s) and equipment to use, (5) how to interpret the results, (6) the limits of the test, and (7) specific actions required when a preparation does not meet specifications. Investigative and corrective action should extend to other preparations that may have been associated with the specific failure or discrepancy. Testing may involve one or more quality attributes, and each test will have one or more acceptable procedures, usually with well-defined acceptance criteria.

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The goal in testing is to determine accurately the adequacy of the compounding process and the quality of the preparation. Any testing procedure used should have accuracy, reproducibility, and specificity. No single testing procedure is suitable for all drugs or preparations because a number of factors determine the validity and reliability of results.

Compounding professionals have two options for the testing that is required for compounded preparations or their ingredients. Some testing methods can easily be performed at the compounding site, but some may need to be outsourced to a contract laboratory. Some testing methods can be conducted in-house by an individual who possesses a good understanding of pharmaceutical analysis and proper training. See *Table 1* for a list of compendial testing methods and *USP* chapters for reference.

Table 1. U.S. Pharmacopeia Chapters for Selected Quality Testing Methods and Procedures

	Chapter Title	Chapter
General Testing		
Boiling point	Distilling Range	⟨721⟩
Density	Density of Solids	⟨699⟩
Ion selective potentiometry	—	—
Loss on drying	Loss on Drying	⟨731⟩
	Pharmaceutical Calculations in Prescription Compounding	⟨1160⟩
Melting point	Melting Range or Temperature	⟨741⟩
Osmolality and osmolarity	Pharmaceutical Calculations in Prescription Compounding	⟨1160⟩
	Osmolality and Osmolarity	⟨785⟩
Particle size	Powder Fineness	⟨811⟩
Particulate matter in injections	Particulate Matter in Injections	⟨788⟩
pH	pH	⟨791⟩
Refractive index	Refractive Index	⟨831⟩
Viscosity change	Viscosity—Capillary Methods	⟨911⟩
Volumetric	Prescription Balances and Volumetric Apparatus	⟨1176⟩
Weight	Prescription Balances and Volumetric Apparatus	⟨1176⟩
Spectroscopy		
Atomic absorption spectroscopy	Atomic Absorption Spectroscopy	⟨852⟩
Fluorescence spectroscopy	Fluorescence Spectroscopy	⟨853⟩
Mid-Infrared spectroscopy	Mid-Infrared Spectroscopy	⟨854⟩
Ultraviolet/visible spectroscopy	Ultraviolet-Visible Spectroscopy	⟨857⟩
Chromatography		
Column chromatography (CC)	Chromatography	⟨621⟩
Gas chromatography (GC)	Chromatography	⟨621⟩
High-performance liquid chromatography (HPLC)	Chromatography	⟨621⟩
Paper chromatography (PC)	Chromatography	⟨621⟩
Thin-layer chromatography (TLC)	Chromatography	⟨621⟩
Microbiology		
Endotoxin testing	Bacterial Endotoxins Test	⟨85⟩
Microbial limit testing	Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests	⟨61⟩
	Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms	⟨62⟩
Preservative effectiveness testing	Antimicrobial Effectiveness Testing	⟨51⟩
Sterility	Sterility Tests	⟨71⟩

If testing is done at the compounding site, appropriate equipment shall be obtained and qualified either by the manufacturer upon sale or by the compounding professional upon receipt and shall be maintained, calibrated, and used properly. If testing is outsourced, the compounding professional should determine what to outsource, how to select a laboratory, and should develop an ongoing relationship with the laboratories chosen. Contract laboratories shall follow standards set forth in *USP* general chapters, as appropriate, and preferably should be registered with the U.S. Food and Drug Administration (FDA).

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Selection of a Testing Method

One general consideration in testing procedure selection is the type of information needed, such as quantitative (strength, concentration), semiquantitative (where a tolerance level is involved, as in endotoxin levels), or qualitative (presence/absence testing, including substance identification, sterility). Another consideration involves the physical and chemical characteristics of the analyte, including solubility, partition coefficient, dissociation constant (pKa), volatility, binding, and the quantity present. The testing method selected also depends upon factors such as sample handling/preparation/purification requirements; type of data needed; and accuracy, reproducibility, and specificity required.

The degree of quantitative measurement and specificity must be considered in the verification process. The typical analytical characteristics used in method verification include accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and ruggedness. Generally, the greater the level of accuracy, precision, or specificity required, the more sophisticated and expensive the testing methods needed. The methods used are also governed by the types of instrumentation available and the standards available for comparison.

Pharmaceutical analysis decisions include procedure selection, obtaining a representative sample (the number of preparation units selected to adequately represent the entire formulation, e.g., 10 randomly selected capsules from a preparation of 100 capsules), storage/shipping of the sample, sample preparation for analysis, the actual analysis, data acquisition, data treatment, and interpretation.

The compounding professional is responsible for implementing a program using selected testing methods for the preparations compounded in the facility. *USP* chapters on spectroscopy and chromatography methods are referenced in *Table 1*. Examples of general and microbiological testing methods are discussed later in this chapter. Examples of selected testing methods for bulk substances and various dosage forms (see *Pharmaceutical Dosage Forms* ⟨1151⟩) are shown in *Table 2*.

Table 2. Selected Compendial Testing Methods for Bulk Substances and Various Dosage Forms

	Testing Method[a]													
Bulk Substances and Dosage Forms	Wt	Vol	pH	Osm	RI	Sp Gr	MP	UV/Vis	HPLC	GC	IR	Sterile	Endotoxin	PM
Bulk substances	–	–	+	–	+	–	+	+	+	+	+	–	+[b]	–
Capsules	+	–	–	–	–	–	–	–	+	+	–	–	–	–
Emulsions	+	+	+	–	–	+	–	–	+	+	–	–	–	–
Gels	+	+	+	–	+	+	–	–	+	+	–	–	–	–
Inhalations	+	+	+	+	+	+	–	+	+	+	–	+	+	–
Injections	+	+	+	+	+	+	–	+	+	+	–	+	+	+
Inserts	+	–	–	–	–	+	+	–	+	+	–	–	–	–
Irrigations	+	+	+	+	+	+	–	+	+	+	–	+	+	–
Lozenges	+	–	–	–	–	–	–	–	+	+	–	–	–	–
Nasals	+	+	+	+	+	+	–	+	+	+	–	*[c]	–	–
Ophthalmics	+	+	+	+	+	+	–	+	+	+	–	+	–	+[d]
Otics	+	+	+	+	+	+	–	+	+	+	–	–	–	–
Powders	+	–	–	–	–	–	–	–	+	+	–	–	–	–
Semisolids	+	–	+	–	–	+	+	–	+	+	–	–	–	–
Solutions, nonsterile	+	+	+	+	+	+	–	+	+	+	–	–	–	–
Sterile implant gels	+	+	+	+	+	+	–	+	+	+	–	+	+	–
Sterile implant solids	+	+	–	–	–	–	+	+	+	+	–	+	+	–
Sticks	+	–	–	–	–	+	+	–	+	+	–	–	–	–
Suppositories	+	–	–	–	–	+	+	–	+	+	–	–	–	–
Suspensions, nonsterile	+	+	+	–	–	+	–	–	+	+	–	–	–	–
Tablets	+	–	–	–	–	–	–	–	+	+	–	–	–	–

[a] Wt, weight; Vol, volume; Osm, osmolality/osmolarity; RI, refractive index; Sp Gr, specific gravity; MP, melting point; UV/Vis, ultraviolet/visible spectroscopy; HPLC, high-performance liquid chromatography; GC, gas chromatography; IR, infrared spectroscopy; PM, particulate matter; +, test applicable; –, test not applicable.
[b] Endotoxin testing may be needed for bulk substances used in compounding some sterile preparations.
[c] *, microbial limits (see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩).
[d] Solutions only, not suspensions or ointments.

Sampling Requirements

Before collecting samples for testing, compounding professionals should consider the following factors:

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- Quantity of preparation being compounded, for a specific prescription versus in anticipation of prescriptions routinely received
- Number of samples needed
- Destructive or nondestructive testing
- Appropriate methods of obtaining representative samples
- Physical state of the samples (solid, liquid, or gas)
- Type of container required for collection and storage
- Any special handling and shipping requirements or restrictions (e.g., controlled drug substances, dangerous or hazardous chemicals, flammable or caustic substances, and refrigerated or frozen preparations)

Storage Requirements

Storage requirements for samples must be specified, including type of container, temperature, humidity, and light protection (see *General Notices and Requirements* and the *Containers, Packaging, Repackaging, Labeling, and Storage* section in this chapter).

The effect(s) that any substance has on the compounded preparation that may interfere or alter the results must be known beforehand. When sending a preparation to a contract laboratory, the compounder should provide the complete written formulation so that the laboratory can quickly determine if there may be any interfering substances present.

Data Interpretation Requirements

The collection of raw data from the testing process must be completed accurately. One must ensure that appropriate and valid descriptive statistics (e.g., mean, standard deviation) are used to analyze the data and that the operating parameters of the analytical instruments are well-established. Reference values, if available, should be provided with the analytical results. A description of the analytical controls used by the laboratory is important for documentation, as is the source of reference standards used to establish standard curves.

Personnel Requirements and Considerations

If testing is done in-house, personnel involved in this activity must be appropriately trained and evaluated with documentation of the training and evaluation. If testing is outsourced, the compounder must be assured of the credentials, proper training, and continuing competency activities of the personnel in the contract laboratory.

PHYSICAL TESTING OF DOSAGE UNITS

[NOTE—In this section the terms "unit" and "dosage unit" are synonymous. To ensure the consistency of dosage units, each unit in a batch should have a uniform weight within a narrow range. Dosage units are defined as dosage forms containing a single dose or a part of a dose in each unit. If multiple dose units are compounded in a batch formulation, the total number of units should not deviate outside of ±10% of the theoretical number of units.]

WEIGHT ASSESSMENT

First, zero or tare the balance. During the compounding process intermediate weighing may be necessary to ensure that all substances have been included and weighed accurately.

At the end of the compounding process, for the dosage form and quantity designated, take care to preserve the integrity of each dosage unit during the following assessment procedures. Assume the concentration (weight of drug substance per weight of dosage unit) is uniform. The following are examples of weight assessment.

Hard Capsules

- Zero or tare balance with an empty capsule.
- Accurately weigh each individual filled capsule from a representative sample of the finished batch (for example, a minimum of 5% of total capsules or 10 individual capsules, whichever is less) and record the weight of each finished capsule on the compounding record.
- Calculate the theoretical weight of a finished capsule's contents.
- Compare the actual content weight of each finished capsule in the representative sample with the theoretical weight of a finished capsule's contents.
- Determine if there is a deviation outside ±10% with any weight of a finished capsule's contents and the theoretical weight of a finished capsule, and if so,
  - Review the compounding record to ensure no steps were omitted.
  - Repeat with a larger representative sample of the finished batch (10% of total capsules or 20 individual capsules, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of ±10% is discovered in the second representative sample, then destroy the batch.

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Other Solids (Including Tablets, Suppositories, Inserts, and Lozenges)

- Accurately weigh each individual dosage unit from a representative sample of the finished batch (for example, a minimum of 5% of total tablets or 10 individual tablets, whichever is less) and record the weight of each dosage unit on the compounding record.
- Calculate the theoretical weight of the dosage unit.
- Compare the actual weight of each dosage unit in the representative sample with the theoretical weight of a dosage unit.
- Determine if there is a deviation outside ±10% with any weight of a finished dosage unit and the theoretical weight of a finished dosage unit, and if so,
  - Review the compounding record to ensure no steps were omitted.
  - Repeat with a larger representative sample of the finished batch (10% of total tablets or 20 individual tablets, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of ±10% is discovered in the second representative sample, then destroy the batch.

Semi-Solids (Including Creams, Gels, and Ointments)

- Accurately weigh an empty container and record the weight on the compounding record.
- Fill an empty container with the final compounded preparation.
- Calculate the theoretical weight of the compounded preparation.
- Weigh the filled container.
- Determine if there is a deviation outside of ±10%, and if so, review the compounding record to ensure no steps were omitted. If the deviation cannot be explained, destroy the batch and prepare a new one.

  Additional Quality Assurance Checks Before Packaging Semi-Solids—
  - Visually inspect the preparation for foreign materials and expected appearance.
  - Measure pH, when applicable.

MICROBIOLOGICAL TESTING

Microbiological testing for pharmacy compounding includes sterility, endotoxin, preservative effectiveness testing, and microbial limit testing (see ⟨797⟩).

Sterility Testing

Sterility tests may be conducted using commercial kits or by developing and verifying USP sterility testing protocols. Standards and procedures are explained in ⟨71⟩.

Endotoxin Testing

Endotoxin tests may be conducted using commercial kits or by purchasing the components separately. Endotoxin testing may be performed in-house with appropriate training and experience. See ⟨85⟩.

Preservative Effectiveness Testing

Preservative effectiveness testing may be conducted when preparing a frequently compounded formulation that contains a preservative. When such a test is performed, the results shall support the beyond-use-date (BUD) assigned to the compounded preparations. See ⟨51⟩.

Microbial Limit Testing

Microbial limit testing may be conducted to provide an estimate of the number of viable aerobic microorganisms (see ⟨61⟩) or to demonstrate freedom from designated microbial species (see ⟨62⟩).

CLEANING, DISINFECTING, AND SAFETY

This section applies to both equipment and facilities (see ⟨795⟩, ⟨797⟩, and *Disinfectants and Antiseptics* ⟨1072⟩).

Change to read:

CONTAINERS, PACKAGING, REPACKAGING, LABELING, AND STORAGE

For storage, packaging, repackaging, and labeling of compounded preparations and repackaging of manufactured products (when defined as compounding in *USP*), refer to *USP General Notices and Requirements* and the following general chapters:

- *Containers—Glass* ⟨660⟩

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- *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩
- *Plastic Materials of Construction* ⟨661.1⟩
- *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩
- *Elastomeric Closures for Injections* ⟨381⟩
- ▲▲ (CN 1-May-2020)
- *Good Repackaging Practices* ⟨1178⟩
- *Good Storage and Distribution Practices for Drug Products* ⟨1079⟩
- *Labeling* ⟨7⟩
- *Packaging and Storage Requirements* ⟨659⟩
- ▲▲ (CN 1-May-2020)
- *Pharmaceutical Dosage Forms* ⟨1151⟩

OUTSOURCING

[NOTE—This section addresses only the purchasing or selling of compounded preparations from pharmacy to pharmacy, not the outsourcing of analytical testing of compounded preparations.]

For pharmacies that prepare outsourced compounded preparations or repackaged commercial products, documentation of beyond-use dating, as defined previously in the *Documentation* section of this chapter, is required and shall be provided upon request. In addition, documentation of compliance with *USP* chapters ⟨795⟩ and ⟨797⟩ is required and shall be provided upon request.

For facilities that receive outsourced compounded preparations or repackaged commercial products, documentation shall be on file for all BUDs assigned to those preparations or products.

RESPONSIBLE PERSONNEL

The responsibility and authority for a quality assurance program should be clearly defined and implemented. Personnel responsible for the quality assurance program should have the education, training, and experience necessary to perform the assigned functions. Quality assurance personnel should assure that documentation, verification, and testing are performed in accordance with written policies and procedures. If deviations from approved policies and procedures occur, it is the responsibility of the quality assurance personnel to investigate and to implement appropriate corrective action. Documentation of any investigations and corrective actions is the responsibility of the quality assurance personnel. Responsible personnel in the quality assurance program are essential in assuring the safety, identity, strength, quality, and purity of compounded drug preparations.

SUMMARY

A quality assurance program is necessary to ensure the quality of compounded preparations. A sound quality assurance program includes detailed SOPs, documentation, verification, analytical and microbiological testing as appropriate to particular compounded preparations, and responsible quality assurance personnel. Compounding professionals must determine the types of testing and degree of testing that will be a part of their quality assurance program. They also must decide whether to perform testing in-house or outsource it to a contract laboratory.

Add the following:

▲⟨1168⟩ COMPOUNDING FOR PHASE I INVESTIGATIONAL STUDIES

1. INTRODUCTION
    1.1 Scope
    1.2 Background
    1.3 Applicable Regulatory Requirements
    1.4 Additional Considerations
2. PERSONNEL TRAINING
3. BUILDINGS AND FACILITIES
4. EQUIPMENT AND COMPONENTS
    4.1 Equipment
    4.2 Components
5. STANDARD OPERATING PROCEDURES
6. PREPARATION ACTIVITIES
    6.1 Retention Samples
    6.2 Disposition of Unused Materials and Preparations

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7. RELEASE OF INVESTIGATIONAL AGENT/PREPARATION
8. LABELING
9. ESTABLISHING BEYOND USE DATES
10. QUALITY ASSURANCE AND QUALITY CONTROL
11. STORAGE, HANDLING, PACKAGING, AND TRANSPORT
    11.1 Storage
    11.2 Handling
    11.3 Packaging
    11.4 Transport
12. DOCUMENTATION
    12.1 Safety Data Sheets
    12.2 Certificate of Analysis
    12.3 Records Management
APPENDIX

1. INTRODUCTION

1.1 Scope

The objective of this chapter is to guide compounders in the compounding of investigational preparations and placebos that are used in investigational studies, specifically Phase I studies for humans. For the purpose of this chapter, the terms "study" or "studies" are used to refer to investigational studies, specifically Phase I investigational studies or trials. When compounding a preparation, the standards in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, and *Hazardous Drugs—Handling in Healthcare Settings* ⟨800⟩ apply. In addition to these standards, this chapter applies when a compounder is preparing an agent for use as an investigational agent in a Phase I clinical study.

1.2 Background

Investigational studies are biomedical or health-related research studies that follow a predefined protocol to ensure subject protection and data integrity. Before drugs are tested in humans, usually a battery of preclinical animal studies is conducted to provide information about the medication's safety profile and pharmacokinetic parameters. Investigational studies in humans can provide a critical understanding of a medication's safety profile (i.e., adverse drug reactions and drug interactions) and pharmacokinetic parameters, and can provide insight into early indications of therapeutic efficacy. Clinical studies generally are conducted in four phases, each with a different purpose to answer different questions.

1. **Phase I studies:** Researchers test an investigational drug or treatment in a small group of people (generally in the range of 20–80 patients or healthy volunteers) for the first time to evaluate the drug's safety, determine a safe dosage range, understand the pharmacokinetic profile, and identify side effects
2. **Phase II studies:** The investigational drug or treatment is administered to a larger group of people (usually NMT several hundred subjects with the specific disease state to be treated) to see if the drug is effective and to further evaluate its safety
3. **Phase III studies:** The investigational drug or treatment is administered to large groups of people (usually several hundred to several thousand subjects with the disease state of interest plus concomitant medical conditions) to confirm the drug's effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that allows the investigational drug or treatment to be used safely
4. **Phase IV studies:** Post-marketing studies delineate additional information about the drug's risks, benefits, and optimal use

Compounding of investigational preparations may be useful 1) when only a small number of doses are needed to support the study, 2) to evaluate various dosage forms or dosage regimens before choosing one for further study, or 3) to develop age-appropriate dosage forms for certain study populations. Compounding investigational preparations may provide flexibility in evaluating dosing ranges and alternative routes of administration, and may reduce the time and cost to do so while maintaining subject safety and high product quality.

Sponsors of studies involving investigational preparations being developed for commercial use usually provide the investigational agents to be used in Phase I studies. Sometimes, however, investigational agents require additional preparation before administration to patients. For example, a lyophilized powder may require reconstitution, or a vial of investigational agent may need to be combined with a solution for infusion. In some cases, a sponsor may use a compounder to prepare the investigational agent from a bulk drug substance or approved drug that the sponsor supplies. For investigator-initiated studies, the investigator may ask a compounder to prepare the investigational agent from either approved drugs or bulk drug substances that are available from commercial sources.

Virtually all dosage forms can be used in investigational studies (see *Pharmaceutical Dosage Forms* ⟨1151⟩). Investigational preparations may include tablets, capsules, powdered drug substance pre-weighed in unit doses, powdered drug substance in bulk for multiple subjects, solution or suspension in unit doses, solution or suspension in bulk for multiple subjects, sterile solutions for injection or infusion, sterile lyophilized powdered drug substance, powder for inhalation, nasal spray, radio-labeled

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bulk and unit doses, bulk drug substance and excipients for compounding, topical preparations such as creams or lotions, and/or placebos.

The formulation to be used in the study should be based on the questions to be answered by the study and should exclude any ingredient (i.e., excipient) that may adversely affect the patient's response to the investigational preparation.

When a third party (e.g., a compounder outside the sponsor's organization) is involved in an investigational study, the sponsor of the study will supply clinical study documentation that provides guidance to the compounder or contract research organization on how to conduct the study. Examples of clinical study documentation include but are not limited to the study protocol, study manual, compounding logs, investigator brochure, and consent forms. Clinical study documentation should be written to clearly specify the scope, roles, and responsibilities of each party involved, including the responsibility of the compounder to follow the clinical study documentation. If a sponsor initiates a contract with a compounder to perform part or all of the Phase I compounding of an investigational preparation, the sponsor and the compounder are both responsible for ensuring that the Phase I investigational preparation is prepared in compliance with applicable requirements. The sponsor should evaluate the compounder according to their auditing practices to ensure that effective quality control (QC) functions are in place.

1.3 Applicable Regulatory Requirements

Regulatory bodies in many countries specify requirements for the compounding of investigational preparations as part of an investigational drug application. Personnel engaged in compounding of preparations for investigational use must comply with these requirements, which vary according to applicable laws, regulations, and guidelines of the regulatory jurisdiction.

This chapter references ⟨795⟩, ⟨797⟩, ⟨800⟩, and *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩, which provide standards for compounding of sterile and nonsterile preparations. With respect to any provisions in these chapters that are inconsistent with applicable regulatory requirements, compounders must comply with the more stringent requirement(s).

1.4 Additional Considerations

A Phase I investigational agent is experimental by definition, and its safety profile in the study population may be unknown. Therefore, the following are examples of questions compounders should be able to answer before they begin compounding any investigational agent. Compounders should be aware of the potential implications of the answers.

1. Will this be a Phase I study?
2. Will there be an Investigational New Drug (IND) application in place for this investigational agent?
3. Will there be an Institutional Review Board (IRB) approval in place for this investigational agent/preparation?
4. Will there be an established triad relationship between the pharmacist, investigator, and patient?
5. Will all the materials supplied by the sponsor or being used to compound the investigational preparation be appropriate for human use as determined by the study sponsor?
6. Will the necessary checks and balances as described in this chapter be in place to ensure subject safety and the compounding of a quality preparation (e.g., independent verification of calculations)?
7. What is the dosage form and what type of testing criteria is specified for this dosage form?
8. Will the investigational preparation interact with any of the dosing devices that are being used in the study?
9. How will the investigational agent be dosed and is the investigational preparation appropriate for that route of administration?
10. What release testing (e.g., assay, pH) will be required for the investigational preparation?
11. Will this preparation be sent to other sites/facilities/locations participating in the clinical study?
12. Has the sponsor provided clear preparation instructions supported by stability and in-use data?
13. Are the available stability data adequate to support the beyond use date (BUD) of the preparation?
14. Is the investigational agent or any components of the final compounded preparation hazardous as defined by the National Institute for Occupational Safety and Health (NIOSH) criteria (see ⟨800⟩)?

2. PERSONNEL TRAINING

In general, all personnel should have the education, experience, and training—or any combination thereof—to enable each individual to perform their assigned function. In particular, personnel should have the appropriate experience to prepare the Phase I compounded investigational preparation and be familiar with QC principles and acceptable methods for complying with applicable regulatory requirements. Additionally, personnel must be knowledgeable with regard to the standards in ⟨795⟩, ⟨797⟩, and ⟨800⟩, and should be knowledgeable about ⟨1163⟩. In addition to the training described in ⟨795⟩, ⟨797⟩, and ⟨800⟩, training for compounding for investigational studies should be described in standard operating procedures (SOPs), should be documented, and should include, but is not limited to, the following, as appropriate for specific protocols:

- Overview of new drug development
- Investigator obligations in FDA-regulated clinical research
- Managing investigational agents and preparations
- Detection, evaluation, and reporting of adverse events
- Audits and inspections in clinical studies
- Monitoring of clinical studies by industry sponsors

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- Health Insurance Portability and Accountability Act (HIPAA) privacy rules for human subject protection
- IRB roles
- Recruitment for participation in research studies and informed consent
- Good Clinical Practice (GCP)
- Human Subjects Protection (HSP)

3. BUILDINGS AND FACILITIES

Facilities should be properly designed and constructed for compounding of the investigational preparations. The design should include special controls (e.g., separate storage of investigational agents) to ensure that the investigational agents/preparations are not commingled with approved drugs used for treatment. The areas used for labeling (see *8. Labeling*), storage, handling, packaging, and transport (see *11. Storage, Handling, Packaging, and Transport*) should be secure, with restricted access. Facility design and use considerations must comply with ⟨795⟩, ⟨797⟩, ⟨800⟩, and applicable regulatory requirements. Additionally, facilities should comply with *Physical Environments That Promote Safe Medication Use* ⟨1066⟩ and ⟨1163⟩. Sponsors should conduct an audit to ensure compliance. Facilities should consider the value of being accredited by a national accreditation agency or organization.

4. EQUIPMENT AND COMPONENTS

4.1 Equipment

Equipment must meet the standards in ⟨795⟩, ⟨797⟩, ⟨800⟩, and applicable regulatory requirements. Additionally, equipment should comply with applicable standards in ⟨1163⟩ and *Prescription Balances and Volumetric Apparatus Used in Compounding* ⟨1176⟩. A number of technologies and resources are available to facilitate and streamline compounding of investigational preparations. Some examples include:

- Disposable equipment and process aids to reduce cleaning burden and risk of cross contamination
- Commercial, prepackaged materials (e.g., *Sterile Water For Injection*, and presterilized containers and closures) to eliminate the need for sterilization of additional materials
- Closed processing equipment (e.g., robotic compounding systems)

The compounder should consider and understand the impact of drug delivery devices (e.g., infusion tubing, pumps, and syringes) used to deliver investigational preparations on the stability and systemic availability of the investigational agent. Information should be evaluated to ensure that the investigational preparation does not interact with or create stability issues when used with the drug delivery device such that it could impact the safety or effectiveness of the preparation (e.g., investigational agent, excipient, or vehicle binding to the IV tubing).

4.2 Components

For sponsor-initiated studies, all materials (e.g., drug substance, excipients, commercial product, packaging components, and in-process material) will likely be supplied by the sponsor. All materials must comply with the standards in ⟨795⟩, ⟨797⟩, ⟨800⟩, available monographs, and applicable regulatory requirements. Any materials not supplied by the sponsor should be appropriate for human use as determined by the study sponsor.

4.2.1 BULK DRUG SUBSTANCES AND EXCIPIENTS

Compounders should have SOPs in place that describe the receipt, handling, review, acceptance, storage, and control of materials to be used to compound preparations for investigational studies. Receipt records should be maintained, and the active substances and excipients used should be traceable to the individual patient. Bulk drug substances and excipients preferably should be official compendial articles, but noncompendial ingredients and substances may be used if they are approved or provided by the sponsor, are evaluated for safety, and the evaluation is appropriately documented. A Certificate of Analysis (COA) or similar product/substance release document confirming the identity, strength, purity, and quality should accompany the bulk drug substance. For human- and animal-derived material, documentation should include information about sourcing and test results for adventitious agents. All bulk investigational agents and other ingredients should be visually inspected for any physical damage and should be quarantined until examined or tested, as appropriate, before they are released for use. Storage and handling conditions for investigational agents (bulk drug substances), other ingredients (excipients), and the final preparation should be described in SOPs and should be maintained to prevent degradation or contamination and to ensure preparation quality. Temperature, humidity, light protection, and other specifications should be provided by the sponsor or supplier. Investigational agent labeling and other ingredient-container labeling should be displayed prominently and understandably with respect to the requirements for proper storage and retest date. If required, in-package temperature-monitoring devices should be used during transport and their information recorded after the package is received at the study site.

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4.2.2 CONVENTIONALLY MANUFACTURED PRODUCTS AS DRUG SOURCE MATERIAL

Conventionally manufactured drug dosage forms (e.g., tablets, capsules, injectables, or liquids) may be used in investigational studies if they are approved or provided by the sponsor. Examples of compounding with conventionally manufactured dosage forms may include, but are not limited to, reconstitution of injectable preparations, over-encapsulation, and incorporating comminuted tablets into an oral liquid or capsule preparation. Generally, immediate-release tablets should be used. Controlled-release tablets can be used if the sponsor determines they are acceptable after careful consideration of the controlled-release mechanism.

4.2.3 IN-PROCESS MATERIALS

In-process materials include any item or preparation that is prepared in advance and held for use during the compounding of the investigational preparation (e.g., premixes, triturations, stock solutions, primary emulsions, and gel components).

5. STANDARD OPERATING PROCEDURES

Appropriate SOPs should be in place to facilitate the compounding of the investigational preparation, which must follow ⟨795⟩, ⟨797⟩, ⟨800⟩, and any applicable regulatory requirements. The procedures should be written clearly and should contain sufficient detail to allow reproducibility of the compounding process and traceability of materials. In addition, the procedures should take into account the complexity of the process and any risks involved. For instance, weighing and compounding a nonsterile preparation starting with a nonsterile powder is less complex and has fewer associated risks compared to preparing and dispensing a sterile preparation compounded from nonsterile ingredients, which is highly complex and entails high associated risks.

The compounding of Phase I investigational preparations should follow written procedures that provide for the following:

- A record that details the materials, equipment, procedures used, and any problems encountered during compounding. Compounders should retain records sufficient to replicate the compounding process. Similarly, if the compounding of a Phase I investigational preparation is initiated but not completed, the record should include an explanation of why compounding was terminated
- Records for handling, compounding, packaging, storage, and transporting investigational agents and final preparations
- A document that identifies procedures for reviewing, approving, and monitoring investigational agents and final preparations
- A record of changes in procedures and processes used for subsequent batches along with the rationale for any changes

6. PREPARATION ACTIVITIES

Before compounding the investigational preparation, the sponsor-designated person should review the protocol and the intended use of the investigational preparation to ensure that adequate space, facilities, equipment, and trained personnel are available to compound the investigational preparation.

The compounder should do a trial run (compound the investigational preparation in accordance with the compounding record) and have the compounded preparation tested per the sponsor protocol. Based on the results of the trial run, the compounding record may need to be changed and additional trial runs conducted to confirm that a quality preparation can be compounded by the site using the compounding record.

In addition, based on these verification studies, appropriate tests (e.g., measurement of final pH of preparation, weight checking all over-encapsulated products) and acceptable limits should be selected, and all future batches evaluated using these agreed upon tests, which should be incorporated into the compounding record. The finished preparation includes the dosage form, package, labeling, and any other required items. Based on the nature of the final preparation (e.g., simple dilution as compared to a powder-filled capsule), the final preparation should be analyzed for conformance to the specifications provided by the primary investigator or sponsor. Additionally, sufficient quality assurance (QA) measures should be incorporated in the process to ensure that the actual yield matches the theoretical yield of finished preparation or that any deviation is accounted for and documented.

Compounded investigational preparations require: correct ingredients and calculations; accurate and precise measurements; and appropriate formulation, facilities, equipment, and procedures. As a final release check, and after obtaining the results of any release testing conducted in accordance with the compounding record and *7. Release of Investigational Agent/Preparation*, the compounder should review each step of the compounding process in the compounding record to ensure that it was completed appropriately and should examine the finished preparation to ensure that it appears as expected. The compounder should investigate any deviations and discrepancies identified during the release check and take appropriate corrective action. Based on information gathered during the investigation, a decision on the outcome of the final preparation should be made and documented. The decision could range from rejection and disposal of the compounded preparation to the release of the preparation for use.

6.1 Retention Samples

A representative sample from each lot of investigational agent and each lot of compounded investigational preparation (finished preparation in the container used in the investigational study) must be retained and properly stored according to the clinical study documentation following study termination or withdrawal of the IND application. If the clinical study

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documentation does not specify the retention time, the sample should be retained for the length of time determined by the sponsor. Sponsors should have access to signed compounding records and individual retained components of the compounded preparation. These individual components must be kept according to the clinical study documentation and retention policies. The sample should consist of a quantity adequate for the performance of additional testing or investigation if required at a later date.

6.2 Disposition of Unused Materials and Preparations

If permitted, unused investigational preparations can be reallocated from one subject to another or from one site to another in accordance with the sponsor's protocol. Unused investigational agents, excipients, or finished preparations must be accounted for and disposed of in accordance with SOPs and sponsor requirements. The disposition (i.e., dispensed, returned to the sponsor, or destroyed) of all investigational preparations should be documented. Any discrepancies should be noted (e.g., preparation of doses not dispensed or that were in broken or breached containers). At the completion of the study, the sponsor should visit the compounding facility to account for all used and unused supplies of the investigational agent. The sponsor should verify the accountability and note the quantity returned for reconciliation and destruction. The compounding facility should verify the quantity returned for destruction or destroyed on-site, and should complete and sign the necessary forms.

7. RELEASE OF INVESTIGATIONAL AGENT/PREPARATION

The final investigational preparation used for subject dosing should be released according to sponsor procedures, which are usually identified in the sponsor-provided clinical study documentation. Integral to release is the assurance that preparation activities have been conducted in accordance with the appropriate quality requirements and as defined by the sponsor, including receipt, handling, preparation, dispensing, labeling, blinding (when necessary), and storage. Any discrepancies should be documented and discussed with the sponsor to determine possible effects and appropriate steps that should be taken. The sponsor is responsible for approval of the final investigational preparation prior to subject dosing, or the responsibility may be delegated to the designated person according to the clinical study documentation.

Sponsors may require the evaluation of one or more quality attributes (e.g., physical, chemical, and microbiological testing) before the investigational preparation is released. Each release test should include one or more procedures, usually with well-defined acceptance criteria. Investigative and corrective actions associated with any specific failure or discrepancy should be documented. Regardless of the source, each investigational preparation and excipient should have predetermined acceptance criteria.

The sponsor or sponsor-investigator should identify a designated person, which may include the compounder, to be responsible for either implementing an in-house testing program or working with a contract laboratory to confirm performance of appropriate testing methods for the investigational preparations. Results of any testing that is undertaken on an investigational preparation should be shared with and discussed with the sponsor of the study. If testing will be done at the compounding facility, appropriate equipment should be obtained and qualified either by the manufacturer upon sale or by the compounder upon receipt, and should be properly maintained, calibrated, and used. All personnel conducting in-house testing should be trained, skilled, and proficient in the procedure(s) necessary for testing.

If a compounding facility has the necessary equipment, supplies, and personnel who are skilled and qualified, many QC tests can be conducted on-site. Appropriate SOPs should be developed and implemented to ensure that equipment and instruments are working satisfactorily and that preparations are tested properly. Compounders can perform physical QC tests to ensure the uniformity and accuracy of compounded preparations. These tests address individual dosage unit weights (including the average), total preparation weight, pH, and physical attributes such as appearance, taste, and smell.

If testing is outsourced, the sponsor and the designated person identified by the sponsor should determine what to outsource and how to select a laboratory, and should develop an ongoing relationship with the laboratory chosen. Contract laboratories must follow standards set forth in *USP* chapters, as appropriate, and preferably should be registered with the FDA.

Factors to coordinate and consider in testing requirements include:

- Quantity of preparation being compounded according to the clinical study documentation for a specific prescription
- Number of samples needed
- Destructive or nondestructive testing
- Appropriate methods for obtaining representative samples
- Physical state of the samples (solid, liquid, or gas)
- Type of container required for collection and storage
- Any special handling and shipping requirements or restrictions (e.g., controlled drug substances, dangerous or hazardous chemicals, hazardous drugs, flammable or caustic substances, and refrigerated or frozen preparations)
- Sponsor-specified storage requirements for samples including type of container, temperature, humidity, and light resistance (see *Packaging and Storage Requirements* ⟨659⟩)

8. LABELING

The term "labeling" encompasses all the written, printed, and graphic material accompanying the preparation, including information on the immediate container received by the patient. Labeling also includes the instructions to the investigators involved in the study, package inserts, cartons, outer wrapping (if used), and any other materials accompanying the

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investigational preparation. Labeling control is important. Only the required quantity of labels should be printed and all labels should be accounted for. An example label should be retained with the compounding record and should be retrievable.

The label includes all written, printed, or graphic matter on the immediate container received by the patient. Appropriate labels should be selected after consideration of the font type and size as well as the adhesive to be used. The printed label should be legible and should adhere to the investigational preparation container during short-term storage and use. The label adhesive should not come in direct contact with the dosage form (e.g., tablet, capsule), leach into packaging materials, or contaminate the investigational preparation.

There should be complete agreement among all of the labeling materials in terms of the information provided. Information on the label should be verified for accuracy by a second person prior to application of the label to the final packaging of the investigational preparation.

Labeling of investigational agents and preparations should follow applicable requirements of the regulatory agency and sponsor.

The investigational protocol or the compounding facility manual should provide labeling instructions as well as label content. Labels may be provided by the sponsor or may be produced on-site at the compounding facility. If labels are provided by the sponsor, the site may be required by laws, regulations, or guidelines of the regulatory jurisdiction, or internal procedures, to provide additional separate and unique labeling. The compounded investigational preparation should be labeled with a unique identifier that allows traceability and recall, if necessary, and a BUD.

However, in the case of investigational preparations, there may be instances when including this information on the preparation labeling might have an adverse effect on blinding. Regulatory bodies may permit exclusion of control numbers and BUDs from the preparation labeling for blinding purposes, provided this information is made available separately (e.g., to the clinical investigator) in case the blinding or randomization code needs to be broken. An auxiliary label for supplying additional information may need to be affixed to individual compounded investigational preparations or a bag that holds a supply of vials or containers of the same drug strength or concentration. Such labels should supply information that is missing on the preparation label or information that is poorly visible on the label. A highlighter pen can be used to focus attention on key information on the label.

9. ESTABLISHING BEYOND USE DATES

A BUD should be established for compounded investigational preparations by the sponsor. Due to the lack of data on investigational agent stability (e.g., if it is a new chemical entity) or stability of the final compounded preparation, the sponsor may not rely on the default BUDs established in ⟨795⟩ and ⟨797⟩. A contract analytical laboratory can help establish an appropriate BUD by performing either real-time or accelerated stability testing. Stability studies may be ongoing simultaneously with the investigational study. In such situations, the sponsor is responsible for ensuring regular communication with the compounder regarding updated stability data and how those data affect a previously identified BUD. New stability data could lead to an increase or decrease in a previously identified BUD. Additionally, compounders should notify the sponsor of any stability issues which may lead to treatment failures in study subjects. The available data should support material storage for as long as intended during the investigational trial.

10. QUALITY ASSURANCE AND QUALITY CONTROL

A QA program for compounding investigational preparations is important for the integrity of a study. QA encompasses all of the processes and procedures undertaken to ensure that compounded preparations are of the quality required for their intended purposes, and also the proper documentation of all steps taken and data obtained. The effectiveness and suitability of the QA program should be assessed regularly per the facility's SOPs.

QA for compounding investigational preparations ensures the following:

- Compounded investigational preparations are designed and prepared according to the methods and procedures in the clinical study documentation, applicable regulatory requirements, and *USP–NF* standards
- Compounding and control operations are clearly specified and implemented according to the regulatory requirements, and *USP–NF* standards
- Compounded investigational preparations are dispensed only if they have been correctly prepared, verified, and stored in accordance with the procedures and parameters defined by the sponsor
- Adequate measures are in place to ensure that the compounded investigational preparations are released, stored, and handled in such a way that the required quality can be ensured until the BUD
- Required documentation is maintained

A QC program for investigational agents and preparations should address the following five components:

1. Bulk drug substances and other ingredients
2. In-process items
3. Packaging materials (e.g., container and closures)
4. Labels
5. Finished preparations

A designated person should be assigned overall responsibility for the establishment and execution of the quality program. Responsible personnel are essential in ensuring the identity, strength, quality, and purity of investigational agents and their components (see ⟨1163⟩).

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A QA program for compounded preparations may include testing during the compounding process and of the finished compounded preparation as determined by the study sponsor.

Sponsors, or their contract facilities including pharmacies or clinics, should conduct audits according to the study requirements. The audit should verify that the designated person identified by the sponsor is performing quality inspections.

11. STORAGE, HANDLING, PACKAGING, AND TRANSPORT

11.1 Storage

Storage conditions (see ⟨659⟩) in all storage areas for investigational agents and preparations should be carefully monitored and controlled, the data should be documented throughout the entire study process, and any deviations should be handled as specified by the clinical study documentation. Any temperature deviations that are outside the sponsor's indicated storage conditions should be investigated, recorded (with duration), and reported to the sponsor. Electronic monitoring and recording devices are recommended because they can provide a detailed record of storage conditions.

11.2 Handling

All materials used in compounding should be handled appropriately and in accordance with information from the Safety Data Sheets (SDS) when available. When compounding with hazardous drugs, compounders should pay careful attention to all aspects of handling these substances to protect personnel and the environment, and must also comply with ⟨800⟩ and all applicable laws, regulations, or guidelines of the regulatory jurisdiction.

11.3 Packaging

All packaging materials—including the immediate container and closure—should be supplied or specified by the sponsor. Packaging materials should meet *USP–NF* standards and should be sourced and selected based on physical and chemical characteristics and compatibility with the final preparation to avoid possible preparation–container interactions. They should also be accompanied by documentation of their composition and size specifications. The packaging may include, but is not limited to, glass or plastic bottles, metal or plastic caps, paper, cardboard, plastic parts and film, metal foil, drums, cans, tubes, vials, or jars.

The packaging should protect and ensure the stability of the preparation. Specifically, the investigational preparation should be packaged to protect it from alteration, contamination, and damage during storage, handling, and transport (see *Storage and Transportation of Investigational Drug Products* ⟨1079.1⟩). If the investigational preparation is sensitive to temperature fluctuations and will be transported to another facility, consideration should be given to using an in-package temperature monitoring device. If used, the information from this device should be recorded immediately after the package is received at the study site.

11.4 Transport

Distribution and dispensing are potentially the least controllable part of the overall scheme from compounding to administration. If distribution and dispensing occur within the same facility, the potential problems are reduced. However, if distribution and dispensing occur in different locations, and external carriers are used, the potential problems can be substantial, especially if overnight delivery is required or distribution needs to occur during weather extremes.

12. DOCUMENTATION

After study termination, records of investigational agents and preparations must be maintained by the compounding facility according to the sponsor's requirements. This includes records pertaining to the preparation, release, and disposition of each lot of material (e.g., drug substance and excipients) used, as well as source documentation and release testing, as appropriate, for bulk materials. Records pertaining to reference standards, if any, that are used to support investigational agents or preparations must be retained according to study requirements.

12.1 Safety Data Sheets

SDS, when available, should be on-site or should be readily retrievable electronically. They should be reviewed by all personnel who will be working with the compounding materials.

12.2 Certificate of Analysis

A COA, or equivalent document if outside the U.S., should be obtained for active pharmaceutical ingredient(s) (API) and excipient(s) used in the compounding of an investigational preparation, and should be maintained throughout the study. It is recommended that the sponsor supply all clinical materials. If all materials are supplied by the sponsor, the sponsor is responsible for maintaining COAs for the ingredients and supplying these to the compounding facility if requested.

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However, if some or all of the clinical materials are supplied by the compounding facility, a COA or equivalent document should be collected from the supplier of the material being used, and the COA should be maintained by the compounding facility and should be provided to the sponsor at the end of the study if requested.

12.3 Records Management

The required documentation for investigational preparations includes the original study specifications, the compounding records, test results, and the COAs. Sponsors should retain all records for at least 2 years after approval of an IND application, according to the clinical study documentation and applicable regulatory requirements, or, if an IND application is not submitted or approved, for 2 years after discontinuation of shipment and delivery of the investigational agent and FDA notification.

APPENDIX

See *Table A-1* for the list of acronyms included in this chapter.

Table A-1. Acronyms Included in ⟨1168⟩

Acronym	Description
API	Active pharmaceutical ingredient
BUD	Beyond use date
COA	Certificate of Analysis
FDA	Food and Drug Administration
GCP	Good Clinical Practice
HIPAA	Health Insurance Portability and Accountability Act
HSP	Human Subjects Protection
IND	Investigational New Drug
IRB	Institutional Review Board
NIOSH	National Institute for Occupational Safety and Health
QA	Quality assurance
QC	Quality control
SDS	Safety Data Sheets
SOPs	Standard operating procedures

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⟨1174⟩ POWDER FLOW

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index or Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

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ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise as a result of segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

Basic Methods for Angle of Repose

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified on the basis of the following two important experimental variables:

1. The height of the "funnel" through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.
2. The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

Variations in Angle of Repose Methods

In addition to the above methods, the following variations have been used to some extent in the pharmaceutical literature:

- *Drained angle of repose* is determined by allowing an excess quantity of material positioned above a fixed diameter base to "drain" from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.
- *Dynamic angle of repose* is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

Angle of Repose General Scale of Flowability

Although there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr*, which is shown in *Table 1*. There are examples in the literature of formulations with an angle of repose in the range of 40° to 50° that were manufactured satisfactorily. When the angle of repose exceeds 50°, the flow is rarely acceptable for manufacturing purposes.

Table 1. Flow Properties and Corresponding Angles of Repose*

Flow Property	Angle of Repose (degrees)
Excellent	25–30
Good	31–35
Fair—aid not needed	36–40
Passable—may hang up	41–45
Poor—must agitate, vibrate	46–55
Very poor	56–65
Very, very poor	>66

Experimental Considerations for Angle of Repose

Angle of repose is not an intrinsic property of the powder; i.e., it is very much dependent upon the method used to form the cone of powder. The following important considerations are raised in the existing literature:

- The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.
- The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a "common base," which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

* Carr, R.L. Evaluating Flow Properties of Solids. *Chem. Eng.* **1965**, *72*, 163–168.

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Recommended Procedure for Angle of Repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2–4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α, from the following equation:

$$\tan(\alpha) = \text{height}/0.5 \text{ base}$$

COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and the tapped volume of a powder.

Basic Methods for Compressibility Index and Hausner Ratio

Although there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume, V_O, and (2) the final tapped volume, V_f, of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{Compressibility Index} = 100 \times [(V_O - V_f)/V_O]$$

$$\text{Hausner Ratio} = (V_O/V_f)$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\text{Compressibility Index} = 100 \times [(\rho_{tapped} - \rho_{bulk})/\rho_{tapped}]$$

$$\text{Hausner Ratio} = (\rho_{tapped}/\rho_{bulk})$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in *Table 2*•.

Table 2. Scale of Flowability•

Compressibility Index (%)	Flow Character	Hausner Ratio
≤10	Excellent	1.00–1.11
11–15	Good	1.12–1.18
16–20	Fair	1.19–1.25
21–25	Passable	1.26–1.34
26–31	Poor	1.35–1.45
32–37	Very poor	1.46–1.59
>38	Very, very poor	>1.60

Experimental Considerations for the Compressibility Index and Hausner Ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder; i.e., they depend on the methodology used. In the existing literature, there are discussions of the following important considerations affecting the determination of (1) the unsettled apparent volume, V_o, (2) the final tapped volume, V_f, (3) the bulk density, ρ_{bulk}, and (4) the tapped density, ρ_{tapped}:

- The diameter of the cylinder used
- The number of times the powder is tapped to achieve the tapped density
- The mass of material used in the test
- Rotation of the sample during tapping

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Recommended Procedure for Compressibility Index and Hausner Ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously because pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

Basic Methods for Flow Through an Orifice

There are a variety of methods described in the literature. The most common method for determining the flow rate through an orifice can be classified on the basis of three important experimental variables:

1. The type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment.
2. The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.
3. The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

Variations in Methods for Flow Through an Orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Because die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container; however, this appears to complicate interpretation of the results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

General Scale of Flowability for Flow Through an Orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

Experimental Considerations for Flow Through an Orifice

Flow rate through an orifice is not an intrinsic property of the powder. It very much depends on the methodology used. Several important considerations affecting these methods are discussed in the existing literature:

- The diameter and shape of the orifice
- The type of container material (metal, glass, plastic)
- The diameter and height of the powder bed.

Recommended Procedure for Flow Through an Orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the "head" of the powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- Diameter of opening > 6 times the diameter of the particles
- Diameter of the cylinder > 2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, making selection of an appropriate construction material an important consideration.

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For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and to better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

Basic Methods for Shear Cell

One type of shear cell is the cylindrical shear cell that is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly; i.e., material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology is rather time-consuming and requires significant amounts of material and a well-trained operator.

Recommendations for Shear Cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

⟨1176⟩ PRESCRIPTION BALANCES AND VOLUMETRIC APPARATUS USED IN COMPOUNDING

1. BALANCES
    1.1 Mechanical Prescription Balances
    1.2 Electronic Balances
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GLOSSARY

1. BALANCES

Pharmacies that perform materials measurements for compounding and dispensing should have access to a well-maintained National Institute of Standards and Technology (NIST) Class III torsion prescription balance or superior balance (preferably an electronic balance) to weigh masses accurately. The pharmacy should have a set of calibration weights, or the balance should have internal calibration capability to standardize the precision and accuracy of the balance.

For more information regarding standards for weights and balances, see *Balances* ⟨41⟩. The standards in NIST Handbook 44 called "Specifications, Tolerances, and Other Technical Requirements for Weighing and Measuring Devices" may also be useful

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to compounders.[1] Some balances offer digital or direct-reading features and printing capabilities, which may be desirable for ease of use. All balances should be calibrated or verified and tested frequently using appropriate test weights.

1.1 Mechanical Prescription Balances

A Class III (formerly Class A) prescription balance is a device designed to accurately weigh drugs and other substances and materials required in pharmaceutical compounding and dispensing practice.[1] It is constructed to support its full weighing capacity without developing undue stresses, and its adjustment is not altered by repeated weighing of the capacity load. The removable pans or weighing vessels should be of equal weight and shape. The balance should have leveling feet or screws and a built-in means to observe its level. The balance may feature dial-in weights and also a precision spring and dial instead of a weighbeam. A balance that has a graduated weighbeam must have a stop that halts the rider or poise at the zero reading, and the reading edge of the rider must be parallel to the graduations on the weighbeam. The distance from the face of the index plate to the indicator pointer or pointers should be NMT 1.0 mm; the points should be sharp; and when there are two pointers, the ends should be separated by NMT 1.0 mm when the scale is in balance. The indicating components and the lever system should be protected against drafts; the balance lid should permit free movement of the loaded weighing pans when the lid is closed; and the balance must have a mechanical arresting device.

BALANCE TESTING PROCEDURE

A Class III prescription balance meets the criteria in the following four tests using an ANSI/ASTM E617 set of Class 6 or better test weights. A typical apothecary weight set contains weights ranging from 10 mg to 50 g in sizes of 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 1 g, 2 g, 5 g, 10 g, 20 g, and 50 g.

Four tests are required to evaluate the suitability of a Class III prescription balance. A balance is acceptable if it passes all four tests. If the balance fails any test, it should not be used until it has been repaired to meet specifications and retested. The four tests are:

1. Sensitivity requirement
2. Arm ratio test
3. Shift test
4. Rider and graduated beam

Sensitivity requirement test: The following requirements are based on (1) the NIST standard that a 6-mg load must displace the pointer 2 mm,[1] and (2) the absence of a 6-mg weight in a typical apothecary weight set.

1. For balances with pointer scale division marks 2 mm apart, a 10-mg weight on one pan and no weight on the other pan, dial, or rider beam of a leveled balance should displace the pointer 1.5-scale division marks, or approximately 6.7 mg/mark, as observed visually. This same 1.5-scale division mark displacement shall be observed with a 10-g weight on one pan and 10-g plus 10-mg weights on the other pan of a level balance.
2. For balances on which the pointer scale division marks are 1 mm apart, a 10-mg weight on one pan and no weight on the other pan, dial, or rider beam of a leveled balance should displace the pointer 3-scale division marks, or approximately 6.7 mg/two marks, as observed visually. This same 3-scale division marks displacement shall be observed with a 10-g weight on one pan and 10-g plus 10-mg weights on the other pan of a level balance.
3. NLT 0.12 g (120 mg) shall be weighed on a Class III prescription to reduce reading errors of less than one mark and two marks in cases (a) and (b), respectfully, to NMT 5% weighing error.

When a balance has been observed or suspected to have been moved, unleveled, contaminated, or otherwise damaged, misaligned, or misused, then its sensitivity must be tested and confirmed to be accurate.

Arm ratio test: The arm ratio test will determine whether both arms of the balance are of equal length.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the marker plate.
3. Place a 30-g weight in the center of each pan.
4. When the balance comes to rest, record the rest point.
5. If the rest point has changed from the middle of the marker plate, place a 30-mg weight on the lighter side.
6. When the balance comes to rest, this new rest point should either return to or go farther than the middle of the marker plate.

Shift test: The shift test checks the mechanics of the arm and lever components of the balance.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the marker plate.
3. Place a 10-g weight in the center of the left pan and place another 10-g weight successively toward the right, left, front, and back side of the right pan, noting the rest point in each case.
4. In any case where the rest point has changed from the center of the marker plate, add a 10-mg weight to the lighter side.
5. When the balance comes to rest, this new rest point either should return to or go farther than the middle of the marker plate.
6. Level the balance and adjust the balance until the pointer is in the middle of the marker plate.
7. Repeat the procedure with the 10-g weight in the center of the right pan, and vary the position of the 10-g weight on the left pan.

[1] NIST Handbook 44 (2015) Specifications, Tolerances, and Other Technical Requirements for Weighing and Measuring Devices. Section 2.2.0 Scales (http://www.nist.gov/pml/wmd/pubs/h44-11.cfm).

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8. Level the balance and adjust the balance until the pointer is in the middle of the marker plate. Make several observations in which both 10-g weights are shifted simultaneously to off-center positions on their pans (i.e., both toward the inside, both toward the outside, one front and the other back). In any case where the rest point is shifted from the middle of the marker plate, the addition of a 10-mg weight on the lighter side should equalize or overcome the shift.

Rider and graduated beam test: The rider and graduated beam test checks the accuracy of the calibrated dial or rider on the balance.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the index plate.
3. Place a 500-mg weight on the left pan and move the dial or rider to the 500-mg point.
4. When the balance comes to rest, record the rest point.
5. If the rest point has changed from the middle of the index plate, place a 10-mg weight on the lighter side.
6. When the balance comes to rest, this new rest point should either return to or go farther than the middle of the index plate.
7. Follow the same procedure using a 1-g weight on the left pan and the dial or rider on the 1-g point. If the new rest point is shifted from the middle of the index plate, a 10-mg weight to the lighter side should equalize or overcome the shift.

1.2 Electronic Balances

A typical electronic prescription balance is an instrument that provides essential readability for materials weighed within the range of capacities for the balance. The display should have prompts to guide users through the balance function, as well as an output port for printing if necessary. Most balances sold for prescription compounding meet or exceed Class I or II accuracy requirements according to NIST Handbook 44 and come with certificates issued under the National Type Evaluation Program (NTEP) of the National Conference on Weights and Measures.

Calibration/certification of the balance should be performed according to the standard operating procedures of the facility. Many electronic balances contain internal calibration programs that automatically calibrate the balance daily. If there is no internal calibration feature, external calibration may be conducted using a calibration weight, according to the procedure supplied by the manufacturer. Weights for use in calibrating an electronic balance should be kept in a special rigid and compartmentalized box and handled with plastic or plastic-tipped forceps, or gloves that are provided with the weights, to prevent scratching or soiling. These calibration weights should meet or exceed ASTM Class 1 criteria. In addition, there are companies that offer calibration services to certify that the balance is performing adequately. For more information regarding the use of electronic balances, see ⟨41⟩ and *Weighing on an Analytical Balance* ⟨1251⟩.

Change to read:

1.3 Minimum Accurately Weighable Quantity on the Balance

The minimum accurately weighable quantity (MAWQ) is the smallest weight or mass that will produce no greater than a predetermined fraction of error on a properly calibrated, situated, and operated balance. The predetermined weighing error is assigned based on either a professional standard (such as NMT 0.05 or 5% error in the weight of any prescription ingredient) or scientific rigor, for example, NMT 0.005 or 0.5% error in the weight of an ingredient that is in limited supply. The compounder should use professional judgment when assigning the acceptable error for each process.

The formula for determining MAWQ for a typical Class III torsion balance is:

$$\text{MAWQ} = \text{Sensitivity requirement/Acceptable error}$$

Example: Calculate the MAWQ for a Class III torsion balance with a sensitivity requirement of 6 mg and an acceptable error of 5% or 0.05.

$$\text{MAWQ} = 6\ \text{mg}/0.05 = 120\ \text{mg}$$

For electronic balances, the MAWQ is calculated using the linearity or the absolute error over the range of the balance. This value is provided by the balance manufacturer. Note that the balance linearity and the readability of the smallest mass unit may not be the same.

Example: Calculate the MAWQ for an electronic balance with a linearity of 0.002 g and an acceptable error of 5% or 0.05.

$$\text{MAWQ} = 0.002\ \text{g}/0.05 = 0.04\ \text{g or } 40\ \text{mg}$$

▲The MAWQ equation in this section should be used if the linearity of an electronic balance is known. If the linearity of an electronic balance is not known, the minimum weight of an analytical balance equation described in *Weighing on an Analytical Balance* ⟨1251⟩, *Qualification, Minimum Weight* should be used and verified at regular intervals as described in the facility's standard operating procedures (SOPs) or at least annually and after any repairs.▲ (USP 1-May-2019)

2. VOLUMETRIC APPARATUS

An assortment of appropriate volumetric devices should be available to accurately measure fluids and liquids of different volumes and densities. Pharmaceutical devices approved for measuring volumes of liquids, including burets, pipets, and cylindrical graduates marked in metric or metric and apothecary units, are to meet the standard specifications for glass

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volumetric apparatus described in "Specifications and Tolerances for Reference Standards and Field Standard Weights and Measures, 2. Specifications and Tolerances for Field Standard Measuring Flasks".[2] Conical graduates are to meet the standard specifications described in NIST Handbook 44.[1] There are ASTM standards (ASTM E542) for the calibration of laboratory volumetric apparatus that may be useful to compounders as well.[3]

2.1 Selection and Use of Graduates

CAPACITY

The capacity of a cylindrical graduate is the volume at the maximum graduation mark at the specified temperature. Volumes for prescription compounding and dispensing that are measured in cylindrical graduates should be adequate to not exceed 5% error.

CYLINDRICAL AND CONICAL GRADUATES

The error in a measured volume caused by a deviation of ±1 mm in reading the lower meniscus in a graduated cylinder remains constant along the height of the uniform column. The same deviation of ±1 mm causes a progressively larger error in a conical graduate, the extent of the error being further dependent upon the angle of the flared sides to the perpendicular of the upright graduate. A deviation of ±1 mm in the meniscus reading causes an error of approximately 0.5 mL in the measured volume at any mark on the uniform 100-mL cylinder graduate. The same deviation of ±1 mm can cause an error of 1.8 mL at the 100-mL mark on an acceptable conical graduate marked for 125 mL.

A general rule for selection of a graduate for use is to use the graduate with a capacity equal to or just exceeding the volume to be measured. Measurement of small volumes in large graduates tends to increase errors, because the larger diameter increases the volume error in a deviation of ±1 mm from the mark. The relation of the volume error to the internal diameters of graduated cylinders is based on the equation:

$$V = \pi r^2 h$$

V = volume
r = radius
h = height

An acceptable 10-mL cylinder having an internal diameter of 1.18 cm holds 109 µL in 1 mm of the column. Reading 4.5 mL in this graduate with a deviation of ±1 mm from the mark causes an error of about ±2.5%, and the same deviation in a volume of 2.2 mL in the same graduate causes an error of about ±5%. *Table 1* shows the accuracy limits for cylindrical graduates.

Table 1. Tolerance or Accuracy Limits for Class A Cylindrical Graduates[a]

Capacity (mL)	Smallest Graduation Mark Interval (mL)	Tolerance (±mL)[b]	Minimum Volume for 5% Error (mL)[c]
5	0.1	0.05	1.0
10	0.1 or 0.2	0.10	2.0
25	0.2 or 0.5	0.17	3.4
50	1	0.25	5.0
100	1	0.50	10.0
250	2	1.00	20.0
500	5	2.00	40.0
1000	10	3.00	60.0
2000	20	6.00	120.0
4000	50	14.50	290.0

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org. Some brands exceed the ASTM limits; for example, one source of a Class A 10-mL graduate lists the tolerance as ±0.08 mL.
[b] The constant volume error in each measurement.
[c] The minimum volume for N% error = [Tolerance (mL) × 100]/N (%); e.g., for N = 5% for a 10-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

There is an inverse relationship between the temperature and density of liquids. For compounding and dispensing purposes, deviations will be negligible when volume measurements are performed at temperatures NMT 5° from that specified on the particular cylindrical graduate, which is usually 20°. For example, the densities in g/mL of water, ethanol, and glycerin vary,

[2] Harris GL. Specifications and tolerances for reference standards and field standard weights and measures. 2. Specifications and tolerances for field standard measuring flasks. 1996. NTIS Order Number: PB96-178926. National Technical Information Service, Alexandria, Virginia 22312 (http://www.ntis.gov).
[3] ASTM E542-01. Standard practice for calibration of laboratory volumetric apparatus. West Conshohocken, PA: ASTM International; 2012. www.astm.org.

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respectively, from 0.999 to 0.997, 0.791 to 0.785, and 1.265 to 1.262 over the temperature range of 15°–25°. The accuracy of each cylindrical graduate used in pharmacy practice is recommended to be verified as follows, at a measuring temperature of 20°–25° with the assumption that deviations follow a normal or Gaussian distribution:

1. Tare a clean, dry graduate on a properly calibrated balance of adequate capacity, linearity, and readability for the volumes to be measured.
2. Fill the tared, dry graduate identically five or more times on a level surface to the smallest, a mid-range, and the maximum capacity graduation marks with *Purified Water* or deionized water, precluding and wiping spills and splashes from the exterior and interior above the target fill line.
3. Record the weight of each filling.
4. Calculate the mean weight of each set of fillings.
5. Calculate the percent deviation of each weight from the theoretical weight as follows:

$$\text{Percent deviation weight} = \{[\text{Actual weight} - (\text{intended volume} \times 0.9975)]/(\text{Intended volume} \times 0.9975)\} \times 100\%$$

6. Calculate the percent mean deviation weight as follows:

$$\text{Percent mean deviation weight} = \{[\text{Mean actual weight} - (\text{intended volume} \times 0.9975)]/(\text{Intended volume} \times 0.9975)\} \times 100\%$$

Deviations for cylindrical graduates used in compounding and dispensing should not exceed 5.0% for individual weights or 2.5% for mean weights of the corresponding volumes of *Purified Water*. *Table 1* and *Table 2* show that a wider range of volumes can be measured in Class A cylindrical graduates compared to Class B. According to ASTM standards, Class A cylindrical graduates must be marked with the letter "A" to designate compliance with applicable construction and accuracy requirements. Class B cylindrical graduates are the same basic design as Class A cylindrical graduates and are considered to be for general purpose use. However, volumetric tolerances may be up to twice the allowable range for Class A cylindrical graduates.

Table 2. Tolerance or Accuracy Limits for Class B Cylindrical Graduates[a]

Capacity (mL)	Smallest Graduation Mark Interval (mL)	Tolerance (±mL)[b]	Minimum Volume for 5% Error (mL)[c]
5	0.1	0.10	2.0
10	0.1 or 0.2	0.2	4.0
25	0.2 or 0.5	0.34	6.8
50	1	0.50	10.0
100	1	1.00	20.0
250	2	2.00	40.0
500	5	4.00	80.0
1000	10	6.00	120.0
2000	20	12.00	240.0
4000	50	29.00	580.0

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org.
[b] The constant volume error in each measurement.
[c] The minimum volume for *N*% error = [Tolerance (mL) × 100]/*N* (%); e.g. for *N* = 5% for a 5-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

2.2 Medicine Droppers

Medicine droppers meet the specifications in *Packaging and Storage Requirements* ⟨659⟩. Medicine droppers should be used only for qualitative purposes, such as pH adjustment with an acid, alkali, or buffer, and visual identification testing with reagents. Medicine droppers are not approved for volumetric measurements for compounding. Calibrated medicine droppers have markings to guide delivery of the prescribed quantity of medication. Their purpose should be limited to the measurement of a dose for administration.

2.3 Dispensing Bottles

Some dispensing bottles may be supplied by the manufacturer as part of the packaging, whereas others may be selected and supplied by the pharmacist. These containers are not necessarily accurately calibrated and should not be used for measurement during compounding and dispensing unless they are appropriately calibrated by the compounder.

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2.4 Syringes

Syringes are available in a variety of sizes with calibrated increments used for measuring and may be used to accurately measure and deliver a wide range of liquid volumes. For viscous liquids, measurements made with syringes are usually more accurate than those made with cylindrical graduates.

ORAL SYRINGES

Oral syringes are available as a device for accurately providing a dose of liquid medication to a patient. The performance of the syringes may be user dependent. For this reason, it is important that suitable operating procedures are documented and followed, and that operators are specifically trained in the correct use of the instruments. Users should be cautious about relying on manufacturers' performance figures. It is more appropriate to perform calibration, taking into account the variation between different users. Oral syringes provide an improvement in dosing accuracy for viscous medications, compared with medication droppers.

SYRINGE CALIBRATION

Syringe calibration is based on the gravimetric determination of the quantity of water either contained or delivered, and the conversion of this value to true volume at the standard temperature of 20°. At 80% of the nominal syringe volume, 5–10 measurements should be performed.

Calibration procedure:

1. Aspirate and dispense an exact volume of deionized water that is 80% of the nominal syringe volume.
2. Determine the mass of the dispensed water.
3. Calculate the volume of the dispensed water using mass and density.
4. Document the measurement values.
5. Calculate the accuracy (*R*).

Calibration formula:

$$\text{Accuracy } (R) = [(\text{Average value} - \text{target value})/\text{Target value}] \times 100$$

2.5 Pipets

Pipets are thin glass tubes used to deliver volumes <25 mL. The two types of pipets are the single-volume pipet and the calibrated pipet. The single-volume pipet is the most accurate and the simplest to use, but the single-volume pipet is limited to the measurement of a single fixed volume; it is not capable of partial volume measurements.

The calibrated pipet has graduation marks from a point near the tip of the pipet to the capacity of the pipet. In addition to delivering its entire contents, the calibrated pipet can be used to deliver partial volumes with good volumetric precision.

Micropipets generally are used when very small volumes (<1 mL) are required; micropipets are available in a variety of sizes. Each micropipet can be adjusted, usually by turning a dial, to deliver a volume within a limited range. For example, one micropipet may deliver volumes of 0–20 µL, another delivers 0–100 µL, and yet another delivers 0–1000 µL. The pipet selected should provide the greatest accuracy for the volume to be measured. Pipets should be calibrated/certified periodically as specified in the facility's standard operating procedures. Calibrating micropipets is a very specialized process that requires adequate training and appropriate equipment. There are companies that offer contract calibration services to certify that pipets are performing correctly.

2.6 Volumetric Flasks

Volumetric flasks have a slender neck and wide, bulb-like base. They are single-volume glassware and come in a variety of sizes. Only one calibration mark is etched on the neck of the flask. When the flask is filled to that mark, the flask contains the volume indicated on the flask. Volumetric flasks are difficult to use if dissolving solids in liquid because of the narrowness of the neck. If solids are to be dissolved, the flask should be partially filled with liquid, the solid material added and completely dissolved, and then the flask should be filled to the calibration mark.

2.7 Additional Considerations When Using Volumetric Apparatus

The use of volumetric apparatus requires some working knowledge of viscosity, density, surface tension, and adhesion. Each of these properties may affect measurement accuracy. For example, higher-viscosity liquids will be drawn into the vessel at a slower rate, and the operator should allow time for complete filling. Delivery can also be slow, because higher-viscosity fluids travel through the orifice at a slower rate. Density can affect filling and emptying in a manner similar to viscosity. In addition, liquids with very low surface tension may tend to "crawl up" the vessel wall or leak from the tip of a syringe or pipet. Adhesion can affect accuracy by resulting in a slow rate of vessel emptying. The operator should confirm that all of the liquid has been drained from the vessel if performing a quantitative transfer.

Understanding the terms "To Contain (TC)" and "To Deliver (TD)" is important, because they apply to glassware. TC and TD glassware consists of vessels that range from 1 to about 100 mL and are individually marked to indicate whether they are TC or TD vessels. A TC vessel is designed to deliver the entire measured content of the operation, and it may require forced air to expel the final quantity. A TD vessel is designed to deliver the entire measured amount via gravity flow.

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GLOSSARY

Accuracy: The closeness of the displayed weight, as measured by the balance, to the true weight, as known by the use of a calibration weight or weights.

Balance indicator: A combination of elements, one or both of which will oscillate with respect to the other, to indicate the equilibrium state of the balance during weighing.

Capacity: Maximum weight, including the weight of tares to be placed on one pan. The nominal capacity of a prescription scale (Class III prescription balance) is assumed to be one-half apothecary ounce (15.55 g), unless otherwise marked. [NOTE—This is applicable only to scales not marked with an accuracy class. Past and currently available Class III prescription balances typically have a capacity of either 60 or 120 g and bear a label stating such.]

Linearity: The ability to maintain the same sensitivity over the entire weighing range of the balance. This is the constant weighing error in every amount weighed within the capacity range of the balance. This term is used only with electronic balances. Analogous terms are "absolute error" or "linear accuracy". The linearity is determined by the balance manufacturer.

Minimum accurately weighable quantity (MAWQ): The smallest weight or mass that will produce no greater than a predetermined fraction of error on a properly calibrated, situated, and operated balance.

Precision: The reproducibility of the weighing measurement as expressed by a standard deviation. A similar term, "repeatability", is sometimes used in specifications for electronic balances.

Readability: The smallest division at which the balance increments.

Repeatability: An instrument's ability to consistently deliver the same weight reading for a given object, and to return to a zero reading after each weighing cycle. This is tested by repeatedly weighing the same test weight.

Rest point: The point on the index plate at which the indicator or pointer stops when the oscillations of the balance cease, or the index plate position of the indicator or pointer calculated from recorded consecutive oscillations in both directions past the zero of the index plate scale. If the balance has a two-pointer indicating mechanism, the position or the oscillations of only one of the pointers need to be recorded or used to determine the rest point.

Sensitivity requirements (SR): The maximum change in load that will cause a specified change, such as a particular quantity of pointer scale division marks, of the pointer or indicating component of the balance.

Tare bar: An auxiliary ungraduated weighbeam bar with a movable poise. It can be used to correct for variations in weighing glasses or papers.

Weighbeam or **Beam:** A graduated bar equipped with a movable poise or rider. Metric graduations are in 0.01-g increments up to a maximum of 1.0 g.

Delete the following:

▲⟨1177⟩ GOOD PACKAGING PRACTICES

This chapter provides general guidance on packaging considerations for Pharmacopeial preparations that may be stored, transported, and distributed. It describes procedures that should be considered to ensure that proper packaging practices are maintained. It does not affect any applicable requirements under good manufacturing practices, state laws governing pharmacy, the *USP General Notices and Requirements* or monographs, or provisions under approved labeling.

Definitions for storage conditions and packaging are provided in ⟨659⟩ *Packaging and Storage Requirements*. All equipment used for recording, monitoring, and maintaining these temperature and humidity conditions should be calibrated on a regular basis. This calibration should be traceable to national or international standards (see also the general information chapter *Monitoring Devices—Time, Temperature, and Humidity* ⟨1118⟩).

CONTAINERS

The monograph packaging and storage statement specifies that the container (primary package) should meet the requirements under *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and *Containers—Performance Testing* ⟨671⟩, which include the stipulations for determining if a container is "tight" or "well-closed." In most cases, compendial preparations are expected to be packaged in "tight" containers, especially if the article is moisture sensitive. In addition, where necessary, the packaging component should protect the preparation from light, reactive gases, solvent loss, microbial contamination, etc. "Tight" and "well-closed" containers are clearly defined in ⟨659⟩ *Packaging and Storage Requirements*, whereas testing protocol and moisture permeation limits to determine if the container meets either of these definitions can be found in *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and *Containers—Performance Testing* ⟨671⟩ for single-unit and multiple-unit containers.

A packaging system is composed of a container system with its closure. This system may include several layers of protection for the Pharmacopeial preparation along with any sealing devices, delivery devices, labeling, and package inserts. The *General Notices* section also provides definitions for types of packaging systems that contain and protect a Pharmacopeial preparation (e.g., single-unit containers, unit-dose containers, etc.). Stability testing is conducted on the dosage forms packaged in the container–closure system proposed for marketing.

One type of permeation test for multiple-unit containers is described in *Containers—Performance Testing* ⟨671⟩. This test is intended for drug products being dispensed on prescription in vials with a container–closure system. The results of the test reflect the water vapor permeation through the container and through the closure. Limits have been established to define whether a container for dispensing has tight or well-closed characteristics with regard to water vapor permeation. FDA

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recommends that manufacturers perform this test on the container–closure system, although it is not specified in *USP*. In this particular test, the inner seal of the manufacturer's container–closure system is removed prior to testing.

Single-unit containers for capsules and tablets under *Containers—Performance Testing* ⟨671⟩ are measured for water vapor permeation according to the criteria for the four classes of containers (classes A–D).

The *USP* recognizes several official container materials that can be selected on the basis of their properties. Most containers are made of glass or plastic. Glass containers must be evaluated for chemical resistance and light transmission (if indicated) as described in *Containers—Glass* ⟨660⟩. In addition, injectable medication containers should be reviewed according to the section *Packaging and Storage Requirements* ⟨659⟩, *Injection Packaging*. Elastomeric closures should be evaluated separately as described in *Elastomeric Closures for Injections* ⟨381⟩. Plastic containers should be assessed as described in *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩; *Plastic Materials of Construction* ⟨661.1⟩; and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. As articulated in these sections, plastics should undergo testing for light transmission (if appropriate), water vapor permeation (see also *Containers—Performance Testing* ⟨671⟩), extraction physiochemical testing, and biological testing (see also *Biological Reactivity Tests, In Vitro* ⟨87⟩ and *Biological Reactivity Tests, In Vivo* ⟨88⟩). For example, testing water vapor permeation for a PE container is conducted by sealing the container with heat-sealed foil laminate and measuring the water permeation in a humid atmosphere. Given that water vapor does not permeate the foil laminate, this test assesses only the properties of the container. The level of protection provided by a packaging system marketed with a heat-sealed foil laminate inner seal (prior to removal of the foil) is approximated by this test. However, in the case of a PET bottle for liquid preparations, water vapor permeation testing is done by filling containers with water and measuring the water loss rate in a dry atmosphere. Additional testing may be required for certain pharmaceutical dosage forms as well.

The container–closure system for the storage or shipment of a bulk liquid drug substance is typically plastic, stainless steel, a glass-lined metal container, or an epoxy-lined metal container with a rugged, tamper-resistant closure. Qualification of the container–closure system for these types of preparations includes evaluation for solvent and gas permeation, light transmittance, closure integrity, ruggedness in shipment, protection against microbial contamination through the closure, and compatibility and safety of the packaging components as appropriate (see *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩).

Other information on container–closure systems may be found in FDA's *Guidance for Industry: Container Closure System for Packaging Human Drugs and Biologics*, www.fda.gov.

PACKAGING

Packaging for Pharmacopeial articles can be divided into categories according to terminology generally accepted by industry. As mentioned earlier, the *General Notices* section provides some definitions for different types of containers classified by their characteristics and uses. In addition, the ASTM Committee D10 on packaging publishes terminology, practices, test methods, specifications, guides, and classifications for testing and evaluating packaging (see ASTM D99695, "Standard Terminology of Packaging and Distribution Environments"). Under certain rules and guidelines (e.g., such as 49 CFR, Dangerous Goods, and others), however, alternate terminology is used for the components described below. For terminology pertaining to repackaging processes, refer to *Packaging and Repackaging—Single-Unit Containers* ⟨1136⟩.

Primary Container

This container is in direct contact with the Pharmacopeial preparation. The purpose of a primary container, also referred to as an immediate container, is to protect the preparation from environmental hazards during storage and handling. In some cases, the primary container is also a specialized delivery system, such as an aerosol or a metered-dose dispenser (see *Pharmaceutical Dosage Forms* ⟨1151⟩). For the majority of oral dosage forms, the primary container consists of a cap and a bottle or a blister or pouch package that can be made from many different materials, including glass, plastic, single or laminated flexible materials, and metal. All components of the primary container must meet the requirements under 21 CFR for direct food contact and, where applicable, the *USP* requirements under *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and *Containers—Performance Testing* ⟨671⟩. A full description of the primary container is included under the "Container/Closure System" section of the New Drug Application (NDA), Abbreviated New Drug Application (ANDA), or other classes of FDA submissions.

Critical Secondary Container

This container is not in direct contact with the article, but it provides essential product stability protection. For example, a primary container may be packed inside a critical secondary container such as a pouch to provide moisture, gas, light, or microbial protection not afforded by the primary container. A description of the critical secondary container is included under the "Container/Closure System" section of the NDA, ANDA, or other classes of FDA submissions.

Secondary Container

This container encloses one or more primary containers. A secondary container is not always present. If used, it is usually designed for the final market presentation. Secondary containers are often used simply to carry required labeling or to keep individual primary containers together with delivery systems or other add-on features. Secondary containers can also provide protection against damage in the handling and distribution system. The most common secondary container is the standard

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folding carton. Some products, such as syringes, may be placed in trays prior to packing in a carton. Secondary container materials are not included in the container and closure description and require neither stability studies nor prior approval when making a change in the materials used.

Additional Packaging

A wide variety of additional packaging, such as trays and display cartons, may be used to hold primary containers.

Unit of Sale

This may be an individual bottle, a carton containing one or more bottles, or a tray with multiple primary containers. A unit of sale may contain more than one item for individual sale. For example, a display tray may have multiples of a single article or a variety of related articles from a single manufacturer, each intended for individual sale. The individual item intended for sale is referred to as a stock-keeping-unit (SKU). SKUs are distinguished by a discrete National Drug Code (NDC). Over-the-counter (OTC) articles contain a Universal Product Code (UPC) for all SKUs. A prescription SKU may be intended for final consumer use and may not be repackaged by a pharmacy. Such packages, often called "unit of issue" or "unit of use," require child-resistant (CR) packaging as described under 16 CFR 1700, "Poison Prevention Packaging," except for packages exempted by the Consumer Product Safety Commission. The CR feature is typically incorporated by the manufacturer (see *Packaging and Repackaging—Single-Unit Containers* ⟨1136⟩). OTC articles are regulated under the same rule, but only if they contain certain active ingredients above specified limits. Any regulated product shipped via the United States Postal Service (USPS) must meet the USPS rules under 39 CFR 111.

Final Exterior Package

This is typically a corrugated fiberboard box (case) or a wrapper. The shipping case label is affixed to this outermost layer and incorporates all of the bar codes required by the National Wholesale Druggists' Association (NWDA). This final package is normally shipped on pallets to distribution centers, wholesalers, and other large-volume customers. The manufacturer may or may not intend that this package enter the small-package-shipping environment as an individual unit without further protection.

Especially with fiberboard boxes, relative humidity (RH) may have a negative effect on the compression strength of the box, causing loads to shift and potentially damage the article or the outer and inner packaging. Articles stored in refrigerators or freezers, which are environments with high RH, are prone to this type of damage when stacked. The problem may be exacerbated by carton design, stacking pattern, or use of low edge-crush-test corrugated fiberboard. Computer programs are available to determine the acceptable stack height and patterns on the basis of carton weight, style, size, and material. If problems occur, the product manufacturer should be contacted. Source materials and reference information on corrugated fiberboard boxes can be found in the "Fiber Box Handbook" published by the Fiber Box Association.

A wholesaler or other reshipper should not assume that the package received from the manufacturer is suitable for reuse. Many packages are customized for very specific routes and modes of transportation and are not suitable for other applications. Like any other shipping container, insulated cartons and inner protective packaging can be damaged during transit, thus affecting package performance and possibly allowing damage to contents if reused.

ENVIRONMENTAL ISSUES

Packaging materials are regulated by a variety of federal, state, and local rules. In general, most pharmaceutical packaging containers can be recycled within local programs. The use of recycled material in primary containers is governed by the FDA, but it is generally not allowed. Pharmaceutical manufacturers commonly follow the most current Coalition of Northeastern Governors' rules (e.g., Model Toxics in Packaging Legislation) regarding heavy metals in packaging and other environmental issues.

Certain classes of Pharmacopeial articles may require special handling. Such articles include products classified as (1) Dangerous Goods under the Department of Transportation (DOT), state, local, or carrier rules; (2) controlled drugs under the Drug Enforcement Administration (DEA); or (3) scheduled substances under state regulations.

LABELING

The labeling of shipping containers by manufacturers must be in compliance with the pertinent sections of FDA and DOT rules.

Dangerous Goods

The labeling of shipments classified as *Dangerous Goods*, including all information on the bill of lading or airway bill, must follow the instructions provided by the DOT, the International Air Transport Association (IATA), and the carrier. The exterior package must carry all of the required standard symbols for the class of goods, and the shipping container must comply with the performance standards for the articles enclosed. The shipper of record is responsible for compliance with the Dangerous Goods requirements.

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Controlled Substances

When Pharmacopeial preparations that contain DEA-scheduled controlled substances are distributed to a patient directly via the USPS, these articles must be marked and labeled in accordance with USPS Domestic Mail Manual, Regulation Article C023, Section 7.2.▲ (USP 1-May-2020)

⟨1178⟩ GOOD REPACKAGING PRACTICES

INTRODUCTION

This chapter is intended to provide guidance to those engaged in repackaging of oral solid drug products; and the chapter provides information to any person who removes drugs from their original container–closure system (new primary package) and repackages them into a different container–closure system for sale and/or for distribution.

This chapter does not apply to pharmacists engaged in dispensing prescription drugs in accordance with state practice of pharmacy. The pharmacist needs to apply

1. the principal information provided in the USP general information chapters *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩ and
2. other beyond-use date references in *Labeling* ⟨7⟩, *Expiration Date and Beyond Use Date.*

ESTABLISHING EXPIRATION DATE

In the absence of stability data, the following criteria should be considered by repackagers when assigning an expiration date.

Unit-Dose Packaging

1. The original container–closure system of the drug product to be used for repackaging must be received un-opened and show no outward signs of having been previously opened.
2. The unit-dose container–closure system must meet the testing requirements under *Containers—Performance Testing* ⟨671⟩ for either *Class A* or *Class B* containers.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The unit-dose container–closure system must meet or exceed the original container's specification for light resistance.
5. The conditions of storage must meet the storage specifications provided in *Packaging and Storage Requirements* ⟨659⟩. Where no specific storage conditions are specified, the product must be maintained at controlled room temperature and in a dry place during the repackaging process, including storage.
6. The expiration dating period used for the repackaged product does not exceed (1) 6 months from the date of repackaging; or (2) the manufacturer's expiration date; or (3) 25% of the time between the date of repackaging and the expiration date shown on the manufacturer's bulk article container of the drug being repackaged, whichever is earlier.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

Multiple-Unit Packaging

1. A repackager may use the manufacturer's original expiration date without additional stability testing if the drug product is repackaged into an equivalent container–closure system that is at least as protective as, or more protective than, the original system and complies with criteria established for equivalency.
2. The original container–closure system of the drug product to be used for repackaging must be received un-opened and shows no outward signs of having been previously opened.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The conditions of storage meet the storage specifications in *Packaging and Storage Requirements* ⟨659⟩. When no specific storage conditions are specified, the product should be maintained at controlled room temperature and in a dry place during repackaging operations.

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5. The type of container–closure system used for repackaging must be at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system.
6. The container–closure system must meet or exceed the original container–closure system's results for light transmission.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

Change to read:

REFERENCES FOR REPACKAGING REGULATIONS AND GUIDANCES

The references listed below are not meant to be all inclusive: specific repackaging operations may have additional requirements.

- **Food, Drug, and Cosmetic Act**
- **Food and Drug Administration Regulations and Guidances**
  Enforcement Policy: 21 CFR, Part 7
  General Labeling Provisions: 21 CFR, Part 201, Subpart A
  Drug Establishment Registration and Listing: 21 CFR, Part 207.20
  Current Good Manufacturing Regulations: 21 CFR, Parts 210–211
  Special Requirements for Specific Human Drugs: 21 CFR, Part 250
  Controlled Substances: 21 CFR, Part 1300
  Potable Water: 40 CFR, Part 141
  FDA *Compliance Policy Guides,* including the following:
    Sub Chapter 430 Labeling and Repackaging
    Sub Chapter 460 Pharmacy Issues
    Sub Chapter 480 Stability/Expiration Dating
- **Applicable USP Chapters**
  ⟨660⟩ *Containers—Glass*
  ⟨661⟩ *Plastic Packaging Systems and Their Materials of Construction*
  ⟨661.1⟩ *Plastic Materials of Construction*
  ⟨661.2⟩ *Plastic Packaging Systems for Pharmaceutical Use*
  ⟨671⟩ *Containers—Performance Testing*
  ⟨1079⟩ *Good Storage and Distribution Practices for Drug Products*

▲▲ (CN 1-May-2020)

GLOSSARY

⟨659⟩ *Packaging and Storage Requirements* provides definitions related to repackaging. For the purposes of this chapter, a repackager, a contract packager, and an equivalent container–closure system are defined as follows:

Repackager: A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient-specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice Regulations in 21 CFR 210 and 211.

Contract packager: A contract packager is an establishment that is contracted to package or repackage a drug product into a single- or multi-unit container. These containers should meet all of the applicable requirements in this chapter. A contract packager does not take ownership from the manufacturer and generally receives the assigned expiration date from the contractor.

Equivalent container–closure system: This term refers to a container–closure system that is at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system. These values may be determined by the repackager, or they may be obtained from the container–closure vendor for the specific container–closure system under consideration.

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⟨1180⟩ HUMAN PLASMA

SCOPE

This chapter provides a consolidated source of information regarding human plasma, with emphasis on plasma for fractionation. Specifically, the chapter addresses plasma classification and nomenclature; collection and processing procedures required for ensuring product safety; details of specific plasmas; and quality systems relating to plasma collection. The chapter also includes, at the end of the text sections, a glossary; a list of abbreviations used in the chapter; and appendices that provide plasma definitions, donor selection criteria, and testing requirements.

Plasma originating from U.S.-licensed collection facilities provides the major supply for the global plasma derivative market. The U.S. Food and Drug Administration (FDA) regulates the collection and processing of plasma used for further manufacture. Title 21 of the Code of Federal Regulations (CFR) details Good Manufacturing Practice (GMP) requirements and product standards to protect the health of the blood donor and to ensure the safety and efficacy of blood products. CFR regulations are updated periodically, but between revisions, existing regulations may not address the most current issues and scientific developments. Therefore, the FDA periodically publishes guidance documents.[1] (For further information, see *Quality Systems*, below).

This chapter emphasizes U.S. practices, but because plasma and its derivatives are shipped globally, it is important to recognize that there are regional differences in recommendations, requirements, and regulations. Therefore this chapter provides information regarding the European Union (EU), the United Kingdom (UK), and Australia.

OVERVIEW

Composition of Plasma

Plasma constitutes approximately 55% of the total blood volume. It is a clear, straw-colored, complex liquid that is 7% protein, 91% water, and 0.9% mineral salts. The majority (approximately 70%) of total plasma protein is albumin. Additional plasma proteins relevant to fractionation include immunoglobulins, coagulation factors, fibrinolytic proteins, proteases, and protease inhibitors. These constituent plasma proteins can be isolated on the basis of the different solubility characteristics of each protein when subjected to specific conditions of pH, temperature, ionic strength, and ethanol concentration. The major products derived from fractionation are listed in *Table 1*.

Table 1. The Major Fractions and Products from the Cohn Process

Fraction	Product
Cryoprecipitate	Antihemophilic factor (FVIII)
Cryosupernatant	Antithrombin III, factor IX complex
Fraction I	Fibrinogen, factor XIII
Fraction II	Immune globulin G (IgG)
Fraction III	IgA, IgM, prothrombin, plasminogen
Fraction IV-1	Factor IX complex, activated factor IX complex
Fraction IV-4	Plasma protein fraction, alpha-1 proteinase inhibitor
Fraction V	Albumin

Plasma for Manufacture of Derivative Products

The two methods for collection of human plasma are automated apheresis (for definitions, see the *Glossary* preceding the appendices) and centrifugation of whole blood donations. Source Plasma collected by apheresis constitutes the majority of plasma used in the manufacture of plasma derivatives in the United States. Plasma collected for transfusion but not so used (i.e., recovered plasma) also may be used for manufacture. Flow charts delineating how apheresis and whole blood–derived plasma can be used in the manufacture of plasma derivatives in the United States and Europe are presented in *Figures 1* and *2*, respectively.

[1] FDA. Blood Guidances. www.fda.gov/cber/blood/bldguid.htm. FDA Memoranda to Blood Establishments available at www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsforManufacturers/MemorandumtoBloodEstablishments/default.htm.

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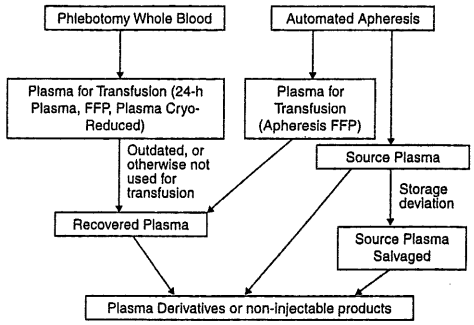

Figure 1. U.S. plasma derivative manufacture: FDA standards.

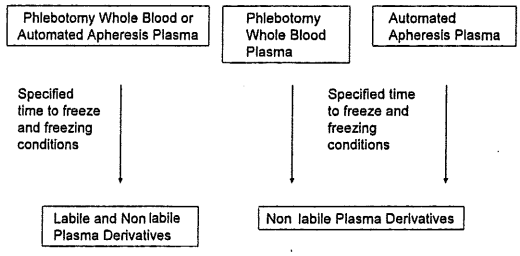

Figure 2. EU plasma derivative manufacture: EU standards.

Regardless of the collection method, plasma for fractionation should be a clear to slightly turbid liquid without visible sign of hemolysis; it may vary in color from light yellow to green; it should be ±10% of the stated volume; and it should show no sign of clots.

Source Plasma

Licensed Source Plasma may be manufactured only in collection centers that are approved by the FDA for the collection and distribution of Source Plasma in interstate commerce. Currently, federal regulations governing the manufacture of Source Plasma, including minimal requirements for donors, are found in 21 CFR 640, Subpart G. By definition, Source Plasma is plasma intended for further manufacture. Source Plasma donors can donate as often as twice a week and may be compensated. In addition to FDA requirements, most plasma collectors and fractionators also comply with voluntary standards established by the Plasma Protein Therapeutics Association (PPTA), a trade and standards-setting organization.[2] PPTA voluntary standards address several areas of donor, plasma unit, plasma pool, and center management, and are designed to supplement existing regulatory requirements.

Plasma for Transfusion

Plasma for transfusion is not intended for further manufacture but for direct transfusion to patients. It may be collected by either whole blood or apheresis donation. In the United States, plasma for transfusion comes from unpaid volunteer donors. Blood collecting facilities that collect plasma for transfusion typically comply with requirements of both the CFR and a voluntary trade organization, the American Association of Blood Banks (AABB). Currently, sections 640.3 and 640.31 of 21 CFR outline requirements for donors of whole blood and therefore govern most donors of plasma for transfusion, regardless of the collection method. AABB voluntary standards include information contained in FDA regulations and guidance pertaining to blood and plasma, as well as additional standards.[3] Plasma for transfusion may be converted to recovered plasma, an unlicensed product that may be used for further manufacture.

Plasma for Ancillary Use in Biologics Manufacturing

Human plasma and its derivatives are used in the manufacture of other biologic products. In this role, the plasma or plasma derivative falls into the category of ancillary use. This is defined as use of a reagent or material as a processing or purification aid or a reagent that exerts an effect on the therapeutic substance but is not intended to be part of the final product formulation (see the *USP* general information chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩).

Human plasma is commonly used in manufacturing processes that involve primary human cells or cell lines intended for therapeutic applications. In these applications, plasma provides a source of protein and possibly other factors that enhance expansion and differentiation of cell populations. A variety of methods have been used to prepare human plasma and derivatives for ancillary use, but the practices are not standardized. Allogeneic plasma typically is obtained from either apheresis or whole

[2] PPTA. www.pptaglobal.org.
[3] AABB. *Standards for Blood Banks and Transfusion Services*. 25th ed. Bethesda, MD: AABB; 2008.

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blood, using citrate anticoagulation. Allogeneic plasma typically is collected from paid donors who, like Source Plasma donors, have been screened for the absence of transfusion-transmissible diseases. Preferred donors may be blood type group AB, because they lack anti-A and anti-B isohemagglutinins. Other preferred donors include untransfused males, because this group is unlikely to have human leukocyte antigen (HLA) antibodies that could react with cells in a given culture system. Serum is prepared either from nonanticoagulated whole blood that has been allowed to clot or by the addition of calcium to plasma obtained from citrated whole blood or apheresis. Heating plasma or serum to 56° inactivates heat-labile complement and other proteins.

Although there are no standardized specifications for plasma products used as ancillary materials, assays often include safety testing associated with general biologics (e.g., bacterial/fungal cultures, endotoxin, and mycoplasma). In addition, characterization of the products may include tests for irregular erythrocyte and HLA antibodies, osmolality, pH, total protein and immunoglobulin concentrations, hemoglobin concentration, and chemistries such as Na, K, Cl, Ca, and glucose.

In some instances, plasma from bovine sources has been used instead of human plasma. The differences between bovine and human plasma products include factors relevant to their efficacy as ancillary materials, as well as safety of the final manufactured product when administered to humans (see the *USP* general information chapter *Bovine Serum* ⟨1024⟩). Fetal bovine serum is preferred to human serum for some applications because it may be superior in promoting human cell growth in vitro. Safety considerations, especially the risk of transmissible spongiform encephalopathy and allergic reactions related to antibovine antibodies, have led to recommendations that human plasma sources be used whenever possible instead of bovine sources. However, substitution of human for bovine plasma does not completely eliminate the risk of infectious and immunologic sequelae, even when human plasma is used as an ancillary material.

PLASMA COLLECTION AND PROCESSING

This section discusses the standard principles involved in plasma collection and the methods used to ensure the safety of the plasma and subsequently manufactured plasma derivatives. Principles for screening and testing of donors are presented in other parts of this chapter.

Collection

Source Plasma is collected by apheresis. Recovered plasma can be obtained either by apheresis or as a by-product of whole blood collection. Collection should take place via an FDA-approved, closed, sterile, pyrogen-free collection system that contains an anticoagulant. No antibacterial or antifungal agent should be added to the plasma. Donations must be collected aseptically. The skin of the donor must be aseptically prepared. Source Plasma is collected using 4% sodium citrate as the anticoagulant. The composition of sodium citrate is given in *Table 2*.

Three anticoagulant solutions are licensed in the United States for collection of whole blood: citrate phosphate dextrose (CPD), citrate phosphate double dextrose (CP2D), and citrate phosphate dextrose adenine (CPDA-1). The composition of blood collection bags containing these anticoagulants is shown in *Table 3*. Plasma for transfusion or further manufacture can be made from a unit of whole blood collected in any of the three anticoagulant solutions. Regulations relating to plasma make no distinction among the three anticoagulant solutions. Consequently, collection, storage, and transport requirements are identical regardless of the anticoagulant solution used in the primary collection.

Table 2. Anticoagulant Solution for Collection of Source Plasma by Apheresis (4% Sodium Citrate)

Volume	Sodium Citrate Dihydrate	Citric Acid Anhydrous	pH (25°)	Ratio of Solution to Whole Blood
250 mL or 500 mL	40 g/L	As required for pH adjustment	6.4–7.5	1:16

Table 3. Anticoagulant Solutions Used during Whole Blood Collection for Recovery of Plasma (500-mL collection bags)

Anticoagulant	CPD[a]	CP2D[b]	CPDA-1[c]
Volume (mL)	70	63	63
Dextrose (mg)	1780	3220	2010
Sodium citrate dihydrate (mg)	1840	1660	1660
Citric acid anhydrous (mg)	209	206	206
Monobasic sodium phosphate (mg)	155	140	140
Adenine (mg)	—	—	17.3
pH (25°)	5.3–5.9	5.3–5.9	5.3–5.9
Ratio of solution to whole blood	1.4:10	1.4:10	1.4:10

[a] Citrate phosphate dextrose.
[b] Citrate phosphate double dextrose.
[c] Citrate phosphate dextrose adenine.

Note: Collection of Source Plasma typically involves the use of sodium citrate as the anticoagulant. The specification for sodium citrate is given in *Table 2*. Plasma for transfusion is stored at 2° to 8° after collection. Plasma collected by apheresis should be frozen immediately at –18° or colder.

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Labeling

The labeling for Source Plasma should comply with 21 CFR 640.70 and 21 CFR 640.69(b). The labeling for whole blood should comply with 21 CFR 606.121 and 606.122, and with internal licenses.

A unique identification number is assigned so that the donation can be related to the individual donor records and test results. The origin of each donation in a plasma pool and the results of the corresponding donation and laboratory tests must be traceable while the required degree of confidentiality concerning the donor's identity is maintained. Whole blood must be labeled "This Product may transmit infectious agents" [21 CFR 121(c)(9)]. Source Plasma or recovered plasma must be labeled "Caution: For Manufacturing Use Only" if the product is intended for use in fractionation. For plasma to be used as a reagent or for in vitro use, the required labeling statement is "Caution: For Use in Manufacturing Noninjectable Products Only" [21 CFR 121(e)(5)(ii)].

Storage

Plasma for fractionation should be stored at or below −20°. The plasma can still be used for fractionation if its temperature exceeds −20° on (at most) one occasion for not more than 72 hours and if the plasma has been maintained at a temperature of −5° or lower at all times. Storage temperatures must be maintained during transport.

PLASMA SAFETY CONSIDERATIONS

Plasma is protected by five overlapping safeguards that the FDA has termed the "Five-Layer Safety Net": donor screening, blood testing, donor deferral, quarantine, and investigation. For guidance in this area, see FDA Publication No. FS 02-1 February 2002.

Voluntary measures that provide an additional margin of safety include recruitment and retention of suitable donors and inventory hold procedures. In the manufacture of plasma-derived products, steps taken for viral clearance are very important for ensuring safety.

None of these measures is sufficient by itself; the safety net is the overlapping combination of the activities.

Donor Screening

The selection of a suitable site for blood and plasma donation activities is a first and very important step to ensure safe donations. Areas with low disease prevalence are preferred as locations for donation centers, thereby reducing the likelihood of collecting plasma from an infected donor.

One of the PPTA voluntary standards is the viral marker standard, which obliges plasma centers to report viral marker rates for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) in the donor populations. A center's rates are compared to the industry average. Alert limits are set to take into account the number of annual donations in the center. If a center exceeds the limit for any of these viruses or the aggregate of all three, the center must implement corrective actions that will bring the center into compliance with the standard.

Appropriate donor selection helps provide a safe plasma supply. A detailed donor history questionnaire in conjunction with a careful medical examination allows center personnel to recognize unsuitable donors whose behavior puts them at risk for transfusion-transmitted disease or who have underlying medical conditions that preclude donation.

An additional measure put in place by centers that collect Source Plasma is the PPTA National Donor Deferral Registry (NDDR). It lists donors throughout the United States who have been previously deferred from donation (although it provides no information about the reason for deferral). Other countries have different systems depending on their national regulations concerning personal data gathering. Any individual who tests positive for HIV, HBV, or HCV is entered into the national database (the National Donor Deferral Registry) used by all U.S. plasma centers that are certified under the International Quality Plasma Program (IQPP). All individuals who present at a U.S. plasma center for the first time are checked against the NDDR. In this manner, donors who have previously been deferred for positive test results at any participating facility can be identified and rejected quickly. This standard ensures that donors deferred for positive test results do not donate in other facilities.

A voluntary safety initiative, the Qualified Donor Standard, implemented by the plasma fractionation industry, builds on the fact that many plasmapheresis donors contribute plasma frequently. A donor who enters a plasmapheresis center for the first time is called an Applicant Donor, and the first donation is used for further manufacturing only if the donor returns a second time. Potential donors must pass two separate medical screenings and testing for HIV, HBV, and HCV on two different occasions. Only after satisfactory screenings and negative test results does that person become a Qualified Donor. If a donor does not return within 6 months, that person loses his/her Qualified Donor status and must qualify again. This standard means that plasma from a one-time-only donor (even when all test results are negative) cannot be used for further manufacture. This standard results in committed donors and eliminates the risk that plasma centers will accept so-called test seekers. The interval between permitted donations of whole blood is too long to allow a similar screening program, although quite a number of donors in whole-blood donor centers are regular and repetitive donors.

Another PPTA voluntary standard addresses donor management criteria. The Community-Based Donor Standard allows only donors who permanently reside within its defined donor recruitment area to donate at a given center. In addition, a Donor Education Standard requires new donors to engage in an educational program and follow-up assessment regarding HIV/acquired immune deficiency syndrome (AIDS) and activities that place them at risk for HIV/AIDS.

In addition to donor management strategies and standards, PPTA has issued a plasma unit management standard called Inventory Hold. This standard states that collected plasma will be held in inventory for at least 60 days from the time of collection. This allows the retrieval of units as a result of post-donation information (information that was not known at the time of donation)

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that would have disqualified the donor. This information could include admitting high-risk behavior; becoming reactive for HIV, HBV, or HCV; or providing incorrect information about international travel.

Blood Testing

Testing of donations is an important safety measure both for plasma intended for transfusion and plasma intended for further manufacturing. Both enzyme-linked immunosorbent assays (ELISA) and nucleic acid amplification technologies (NAT) are used to screen donations for the presence of infectious disease. Automation provides the necessary throughput to screen every donation for a variety of potential pathogens.

Testing strategies differ depending on whether the plasma donation is intended for transfusion or further manufacture. Plasma for transfusion requires more extensive infectious disease testing, because there are no pathogen inactivation/removal technologies licensed for this product in the United States. On the other hand, plasma for further manufacture is subjected to several pathogen inactivation/removal steps during manufacture, thereby obviating the need for some disease testing. *Table 4* outlines current infectious disease tests required by the FDA for plasma donations collected in the United States. *Appendix 3* compares EU and U.S. disease testing and donor deferral requirements.

Table 4. FDA Disease Test Requirements for Plasma for Transfusion and Plasma for Further Manufacturing

Disease	Plasma for Transfusion	Plasma for Further Manufacturing[a]
Hepatitis B	Hepatitis B surface Antigen (HBsAg) Hepatitis B core antibody	HBsAg
Hepatitis C	Anti-HCV HCV RNA	Anti-HCV HCV RNA
HIV	Anti-HIV I/II HIV RNA	Anti-HIV I/II HIV RNA
Human T-lympho-tropic virus (HTLV) I/II	Anti-HTLV I/II	Not required
Syphilis	Serologic test for syphilis, every donation	Serologic test for syphilis, every 4 months for donors only
West Nile virus (WNV)[b]	WNV RNA	Not required

[a] The FDA also encourages in-process NAT testing for parvovirus B19 and hepatitis A. HBV NAT testing also is performed on most Source Plasma.
[b] Testing for WNV is recommended in an FDA draft guidance. The FDA is considering recommendations regarding testing for *Trypanosoma cruzi* (Chagas disease).

NAT, of which polymerase chain reaction (PCR) is the most widely used form, does not rely on the detection of antibodies produced by the infected host after exposure, but targets the nucleic acid of the infecting agent. By means of the selection of suitable priming molecules (the so-called primers), the assay is highly specific for the infecting virus (see the *USP* general information chapter *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩). Through several cycles of amplification, the polymerase enzyme can repetitively generate copies of the targeted fragment of the viral nucleic acid, providing an exponential amplification of a very short stretch of the viral deoxyribonucleic acid (DNA) [or ribonucleic acid (RNA)]. The exponential amplification leads to the generation of many copies of the target molecule and allows the subsequent detection of this virus-specific fragment, even if the original viral load was exceedingly low. This methodology has brought a new degree of safety.

NAT testing, because of its complexity and expense, is difficult to conduct on individual donations. Generally, aliquots from several donations are combined into a single pool, often called a minipool. Testing in pooled format remains more sensitive than serological ELISA screening of individual donations. In addition, the NAT principle circumvents several of the limitations in detecting pathogens by means of serological methods. Pooling can influence overall sensitivity, depending on the pool size and the analytical sensitivity of the NAT assay employed.

In many countries, the maximal load of a pathogen acceptable for a single donation defines the overall NAT sensitivity required. Assays of higher analytical sensitivity can use larger pools, but those of lower analytical sensitivity must test smaller pools in order to comply with regulations. The availability of commercial NAT test kits with defined analytical sensitivity has made minipools up to 512 very common, because these pool sizes, in combination with the analytical sensitivity of the assays used, comply with common regulations on overall sensitivity.

Effective NAT screening requires that the viral load of the plasma pool at the beginning of production be less than the inactivation and/or removal capacity of the process. Differences between the plasma transfusion and fractionation industries have led to different applications of NAT. For individual donations intended for transfusion, where there is no inactivation and/or removal process and where testing is the only method to interdict a contaminated donation, the safety of each individual donation must be ensured by testing with the most sensitive assays possible. Plasma intended for further manufacturing, in contrast, is pooled and serves as the starting material for a multistep process that has built-in pathogen inactivation methodologies. Therefore, NAT screening for plasma for further manufacture is focused on ensuring safe donations and limiting the viral load of the plasma pool to levels less than the known viral inactivation/removal capacity of the inactivation process.

To avoid the loss of large amounts of plasma from a reactive pool, the fractionation industry has implemented a prescreening strategy, the minipool screening concept mentioned earlier. Aliquots of plasma donations are combined to form minipools, and the minipools are tested by NAT. If a minipool is reactive for a virus tested, the individual donation that gave rise to this positive result can be identified and interdicted. The other donations demonstrated to be free of infection can be used for further

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manufacturing. The donations are then combined into a production pool, a sample of which is subjected to NAT testing as required by regulations.

As indicated, the NAT test portfolio is not uniform and depends on the intended use of the donation and the regulatory environment. Although screening for HCV RNA is done in most countries, screening for HIV is not universally required. NAT detection of HBV is used mainly for plasma for manufacturing. Screening for B19 virus or hepatitis A virus (HAV) is performed only on plasma for manufacturing. HBV screening using NAT detection is more widespread in European and Asian countries than in the United States.

Donor Deferral

A donor may be deferred from further donation as a result of answers provided on the donor history questionnaire, counseling of donors for reasons for deferral, a medical examination performed at the time of donation, or positive tests for infectious diseases. These processes ensure both the eligibility of the donor and the suitability of the donation. In the event that either is not acceptable, standard processes permanently remove the donor and interdict unused units previously donated. The donor registries mentioned earlier are one means of ensuring that a donor deferred at one center cannot donate elsewhere.

Quarantine and Inventory Hold

Each individual unit of plasma, whether for transfusion or further manufacture, is held in quarantine until all the required tests have been completed. If all required tests have been performed and found acceptable, the unit can be released; if not, the unit must be destroyed. The plasma industry has voluntarily implemented the inventory hold protocol (also discussed in the previous section *Donor Screening*) for plasma for further manufacture. According to inventory hold requirements, during a 60-day hold period an individual plasma donation cannot be used for further manufacture. The rationale for the hold is that donors who have been recently infected with a pathogen may not have developed levels of antibody at the time of donation, thereby donating an infectious unit despite negative disease tests. The hold provides sufficient time for an infectious donor to develop levels of antibody that will be detected during a subsequent donation if the plasma was intended for transfusion. The 60-day hold also reduces the chance of releasing an infectious unit into the manufacturing process.

The introduction of NAT may have decreased the need for inventory hold, because NAT targets the infecting virus directly and thus does not rely on the time-delayed production of antibodies. Because NAT cannot detect all viruses and because even NAT has a certain (although very low) limit of detection, inventory hold is still of value and thus remains in place in the plasma fractionation industry.

Investigation

Each plasma donation must be traceable from donation to ultimate disposition in order to minimize the potential transmission of an infectious agent. Traceability encompasses all data concerning donation site, donor identifying information, test results, and data regarding transport, storage, and consignee(s).

Look-back is a process to identify and interdict (quarantine) previous donations from a donor who, at a subsequent donation event, has been found to be (1) infected with a transmissible agent or (2) unsuitable for donating plasma because of history, physical examination, or post-donation information. Although look-back strategies are similar in most countries, specific procedures may vary.

QUALITY SYSTEMS

The intent of this section is to outline the general principles and regulations that are the basis of quality systems relating to plasma collection. U.S. collection centers must follow cGMPs that originate in CFR and are elaborated in FDA regulations, guidance documents, and industry standards.

The GMP regulations specifically governing plasma are found in 21 CFR 600, Biological Products: General; and 606, Current Good Manufacturing Practice (cGMP) for Blood and Blood Components. More general cGMP regulations are found in 21 CFR 210, Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or General Holding of Drugs, General; and 21 CFR 211, Current Good Manufacturing Practice for Finished Pharmaceuticals. Although quality systems regulations are part of 21 CFR for medical devices, they have been extended to other manufacturing as part of the "c" in cGMP and FDA Guidance documents.[4]

The quality system is divided into four major parts: management responsibility, resources, manufacturing operations, and evaluation activities. These are the foundation of the five manufacturing systems: production, facilities and equipment, laboratory control, materials, and packaging and labeling. The procedures of each system are designed to allow operations that facilitate implementation of cGMP requirements. In many instances, these requirements relate to providing facilities and expertise to achieve the requirements for the Five-Layer Safety Net (donor screening, blood testing, donor deferral, quarantine, and investigation), discussed above.

Management responsibilities include providing leadership; building a quality system for the organization that meets requirements; establishing policies, objectives, and plans; and reviewing the quality systems with defined frequency. Resources include having sufficient resources for operational activities, personnel development plans, adequate facilities, and suitable equipment; and controlling outsourced operations. Manufacturing includes designing, developing, and documenting product

[4] FDA. Guidance for Industry: Quality Systems Approach to Pharmaceutical cGMP Regulations. 2006. Available at www.fda.gov/downloads/Drugs/.../Guidances/UCM070337.pdf.

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and processes, performing and monitoring operations, and addressing nonconformities. Evaluation activities include analyzing data for trends, conducting internal audits, and initiating corrective and preventive actions.

A number of required routine activities related to collection and release of plasma or normal recovered plasma from whole blood are linked to both cGMP guidelines and quality systems. These include the requirement for having SOPs to cover all aspects of collection, testing, and release. It is also necessary to validate equipment and systems used by the collection center, including temperature-controlled areas, laboratory equipment, water systems, and computer systems. The design and operation of the facility must be adequate to perform the tasks at hand and prevent cross-contamination. Plasma collection facilities must have an adequate number of knowledgeable and trained staff as well as procedures for acceptance and release of raw materials. To the extent possible, collection facilities must adhere to the GMP requirements for a pharmaceutical manufacturing facility.

GLOSSARY

Apheresis: A method of obtaining one or more blood components by machine processing of whole blood; the residual components of the blood are returned to the donor during or at the end of the process.

Blood component: A constituent of human blood: red cells, white cells, platelets, or plasma.

Blood establishment: Any structure or organization responsible for any aspect of the collection and testing of human blood or blood components, whatever their intended purpose, and their processing, storage, and distribution. Hospital transfusion services engaged only in compatibility testing and transfusion of blood and blood products are not included within the definition of blood establishment.

Blood product: Any therapeutic product derived from human blood or plasma.

Center: Collection site or location where blood or plasma is collected (and also may be processed and stored). *Center* is also applicable to a testing *Site* (see entry in this glossary).

Cryoprecipitate: A plasma component prepared from fresh-frozen plasma by freeze-thaw precipitation of proteins and subsequent concentration and resuspension of the precipitated proteins in a small volume of the plasma.

Deferral: Temporary or permanent suspension of the eligibility of an individual to donate blood or blood components.

Distribution: The act of delivery of blood and blood components to other blood establishments, hospital blood banks, and manufacturers of blood products.

Manufacturing pool: A combination of a specified number of plasma donations used as the first step in the manufacture of plasma derivatives.

Donation minipool: A combination of a small number of units or samples representative of donations used for pretesting prior to pooling units for manufacture.

Quarantine: The physical isolation of blood components or incoming materials/reagents over a variable period of time while awaiting acceptance, issuance, or rejection of the blood components or incoming material/reagents.

Site: Any location at which a blood establishment carries out blood collection, not including any location not owned or managed by the blood establishment at which blood is collected or any mobile blood collection unit.

Validation: The establishment of documented and objective evidence that the particular requirements for a specific intended use can be consistently fulfilled.

ABBREVIATIONS

AABB	American Association of Blood Banks
AIDS	Acquired immune deficiency syndrome
CFR	Code of Federal Regulations
CJD	Creutzfeldt-Jakob disease
CMV	Cytomegalovirus
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EIA	Enzyme immunoassay
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFP	Fresh-frozen plasma
FP24	Plasma frozen within 24 hours after phlebotomy
cGMP	Current Good Manufacturing Practice
HAV	Hepatitis A virus
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCT	Hematocrit
HCV	Hepatitis C virus

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HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-lymphotropic virus
IgA, IgG, IgM	Immunoglobulins A, G, and M, respectively
IQPP	International Quality Plasma Program
IU	International Unit
NAT	Nucleic acid amplification technology
NDDR	National Donor Deferral Registry
PCR	Polymerase chain reaction
PPTA	Plasma Protein Therapeutics Association
PRP	Platelet-rich plasma
RNA	Ribonucleic acid
SARS	Severe acute respiratory syndrome
TEP	Therapeutic exchange plasma
WBDP	Whole blood-derived plasma
WNV	West Nile virus

APPENDICES

Appendix 1

[NOTE—The collection, processing, and uses of plasma have generated a large number of terms and definitions that reflect the diversity of operations. In addition to the FDA standards and terms, industrywide voluntary standards are discussed in the *Plasma Safety Considerations* section.

Advisory Note: These terms are not meant as regulatory definitions, because plasma term definitions can vary from region to region and among industry sectors. The reader is advised to consult with regulatory authorities responsible for the region and industry sector. Often specific process variables must be considered.]

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions

Plasma Type and Agency or Agency Type	Specification
Recovered plasma	
CFR	Plasma derived from single units of whole blood as a by-product in the preparation of blood components from whole blood collection and intended for further manufacturing. Compliance Policy Guides Manual (CPG 7134.12), Sec. 230.100.
AABB	Plasma for use in manufacturing and prepared from allogenic donations. Plasma selected for manufacture that has been collected from whole blood or apheresis plasma collected for transfusion that has expired.
Inter-region, Inter-sector	Plasma separated from whole blood most often by manual centrifugation or by apheresis. The priority for the blood collected is usually for the production of red blood cells. However, the plasma can be suitable for further manufacture of biotherapeutics and transfusion. The time from collection to freezing can vary depending on the distance of collection and processing sites. Volunteer donors typically are used.
Source Plasma	
CFR	Fluid portion of human blood collected by plasmapheresis and intended as source material for further manufacturing use (21 CFR 640.60).
Inter-region, Inter-sector	Plasma separated from whole blood by plasmapheresis where the cellular components can be returned to the donor. The priority for the plasma usually is for further manufacture of biotherapeutic products. However, the plasma can be suitable for transfusion. It is rapidly frozen after collection.
Fresh-frozen plasma (FFP)	
CFR	Fresh-frozen plasma shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and minimal manipulation of the donor's tissue. The plasma shall be separated from the red blood cells and placed in a freezer within 8 hours or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system and stored at −18° or colder [21 CFR 640.34(b)].
AABB	Plasma separated from the blood of an individual donor and placed at −18° or colder within 6 to 8 hours of collection from the donor or within the timeframe specified by the manufacturer's instructions.
Inter-region, Inter-sector	Plasma that is collected and frozen quickly after preparation. Transfusion is the primary intended use. However, FFP can be suitable for further manufacture of biotherapeutic products.
Council of Europe	A component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain labile coagulation factors in a functional state (Chapter 21).

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Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions *(continued)*

Plasma Type and Agency or Agency Type	Specification
UK	Supernatant plasma separated from a whole blood donation or plasma collected by apheresis, frozen, and stored.
Australia	Plasma, Fresh Frozen is a component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain the labile coagulation factors in a functional state. If prepared from whole blood, it should preferably be recovered within 6 hours, and not more than 18 hours after collection if the unit has been refrigerated. Plasma may also be collected up to 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°. Separated plasma must be frozen to below −30° within one hour. Freezing of plasma collected by apheresis, as above, must commence within 6 hours of collection or within 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°.
Concurrent plasma	
Inter-region, Inter-sector	Plasma collected concurrently with cellular components. Concurrent plasma may be suitable for transfusion or for further manufacture of biotherapeutics.
Applicant Donor	
Inter-region, Inter-sector	Source Plasma obtained during the first collection from a new donor. The plasma is reserved for testing, and any remainder or products derived from the remainder are not allowed for use in humans or are quarantined until the donor passes appropriate tests and returns for a second donation which also clears testing. At that time, both collections are reclassed as "Qualified".
Platelet-rich plasma (PRP)	
CFR	PRP shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and manipulation of the donor's tissue. The plasma shall be separated from the red blood cells by centrifugation within 4 hours after completion of the phlebotomy or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system. The time and speed of the centrifugation shall have been shown to produce a product with at least 250,000 platelets per µL. The plasma shall be stored at a temperature between 20° and 24° immediately after filling the final container. A gentle and continuous agitation of the product shall be maintained throughout the storage period if stored at a temperature of 20° to 24° [21CFR 640.34(d)].
Inter-region, Inter-sector	Plasma that is a product of the first centrifugation of blood where it is separated from red cells. Platelets are fractionated into the plasma layer.
Platelet-poor plasma	
Inter-region, Inter-sector	Plasma that is further purified from platelets by a second centrifugation of PRP.
Cryo-poor plasma	
CFR	Plasma that remains after both platelets and cryoprecipitated AHF have been removed may be labeled "Plasma, Cryoprecipitate Reduced" [21CFR 640.34(e)(2)].
AABB	Plasma Cryoprecipitate Reduced; Fresh-frozen Plasma from which cryoprecipitate has been removed.
Inter-region, Inter-sector	Plasma that has been thawed by maintaining the temperature just above freezing (usually 4°). A large portion of certain plasma proteins (e.g., FVIII, cryoprecipitate, fibrinogen, fibronectin, or FXIII) has been precipitated from the plasma.
Council of Europe	Plasma, Fresh-Frozen, Cryoprecipitate-Depleted (Chapter 23). A component prepared from plasma by the removal of cryoprecipitate.
UK	Plasma cryoprecipitate-depleted for transfusion means a plasma component prepared from a unit of plasma, fresh-frozen. It comprises the residual portion after the cryoprecipitate has been removed.
Australia	Plasma, Fresh Frozen, Cryoprecipitate-Depleted is a component prepared from Fresh Frozen Plasma by the removal of cryoprecipitate. The content of albumin, immunoglobulin, and most clotting factors is maintained, but the levels of Factors V and VIII and fibrinogen are reduced. It can be stored for up to 36 months at below −25°.
Cryo-rich plasma	
Inter-region, Inter-sector	Plasma that has been thawed by gentle heat input (e.g., in a 37° water bath) where the cryoprecipitate remains dissolved.
Plasma for labile products	
Inter-region, Inter-sector	Plasma that has been collected and best maintains the activity and integrity of labile plasma proteins as exemplified by clotting Factor VIII. Generally the time from collection through processing to freezing is rapid.
Plasma for stable products	
Inter-region, Inter-sector	Plasma that has been collected where conditions for preservation of labile products was not achieved, however conditions were sufficiently moderate so relatively stable products like IgG and albumin would not be impacted.
Less than 6-hour plasma	
Inter-region, Inter-sector	This is generally recovered plasma that has been collected, processed, and frozen prior to 6 hours after collection. This plasma generally is considered to be acceptable for the production of labile products.
6- to 12-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 6 hours and less than 12 hours after collection. This plasma is generally considered to be acceptable for the production of labile products but is inferior to less than 6-hour plasma for this purpose.

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Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions *(continued)*

Plasma Type and Agency or Agency Type	Specification
12- to 24-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 12 hours and less than 24 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose.
Less than 12-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen less than 12 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose.
More than 24-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 24 hours after collection and usually less than 72 hours after collection. This plasma generally is not acceptable for the production of labile products.
Pooled plasma	
Inter-region, Inter-sector	Plasma that has been pooled for manufacturing from several donors. Some plasma pools for further manufacture of biotherapeutic products can be derived from several hundred to a few thousand donors.
Single-donor plasma	
Inter-region, Inter-sector	Plasma derived from a single donor. It can be a single unit or a pool of several units derived from multiple collections from the same donor.
Hyperimmune plasma	
Inter-region, Inter-sector	Plasma derived from donors with high titers to specific disease agents. Titers are elevated in these donors mostly as a result of immunization with a vaccine (e.g., hepatitis B, tetanus, or rabies) or exposure to disease agents (e.g., HCV or SARS). Hyperimmune plasma usually is intended for the preparation of IgG to provide passive immunity against target disease agents.
S/D plasma	
Inter-region, Inter-sector	Plasma that has been treated with solvent/detergent, an inactivation method effective against envelope virus disease agents (e.g., HIV, HBV, or HCV). Some plasma protein components are inactivated or damaged by the process (e.g., alpha-1 proteinase inhibitor, Protein S, anti-plasmin, or FVIII).
EU (PharmEuropa)	Human Plasma Pooled and Treated for Virus Inactivation is a frozen or freeze-dried, sterile, nonpyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion. The human plasma used complies with the monograph on Human Plasma for Fractionation.
Therapeutic exchange plasma (TEP)	
Inter-region, Inter-sector	Similar to Source Plasma in its collection. However, the donors are patients who are having their plasma replaced with electrolytes, protein solutions, or plasma from another donor. The objective usually is to remove disease elements from the patient's plasma. Generally, TEP is not advisable for further manufacture of biotherapeutic products. However, there may be cases where a specialty product may propose a specific TEP as a source material.
Quarantine plasma	
Inter-region, Inter-sector	Plasma that has been collected and not had initial testing completed and/or stored as part of a controlled donor program. The donor is retested for disease agents (e.g., 6 months after collection). If the donor again is negative for the tested disease agents, then the plasma is released for use for further manufacture and/or use in humans.
Quarantine residual plasma	
Inter-region, Inter-sector	Plasma that has been collected and stored as part of a controlled donor program. The donor was not retested for disease agents (e.g., 6 months after collection). An example would be that the donor did not return to the collection facility to permit the later test. Quarantine Residual Plasma is not recommended for further manufacture and/or use in humans.
Salvaged plasma	
Inter-region, Inter-sector	Plasma that has experienced a storage or transport temperature deviation but may still be useful for the preparation of nonlabile products such as albumin or IgG.
Plasma for fractionation (Processing requirements)	
CFR	Placed in a freezer within 8 hours and stored at −18° or colder.
Australia	The liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products. When the plasma is intended for the recovery of proteins that are labile in plasma, it is frozen rapidly to −25° or below within 24 hours of collection. For the recovery of nonlabile proteins, the plasma should be frozen to −20° or below as soon as possible and at the latest within 72 hours of collection. Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below −20°. For accidental reasons, the storage temperature may rise to above −20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds −20° does not exceed 72 hours; the temperature does not exceed −15° on morethan one occasion; the temperature at no time exceeds −5°.

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Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions *(continued)*

Plasma Type and Agency or Agency Type	Specification
EU (PharmEuropa)	Human plasma for fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products. When obtained by plasmapheresis or from whole blood (after separation from cellular elements), plasma intended for the recovery of proteins that are labile in plasma is frozen within 24 hours of collection by cooling rapidly in conditions validated to ensure that a temperature of –25° or below is attained at the core of each plasma unit within 12 hours after it is placed in the freezing apparatus. When obtained by plasmapheresis, plasma intended solely for the recovery of proteins that are not labile in plasma is frozen by cooling rapidly in a chamber at –20° or below as soon as possible and at the latest within 24 hours of collection. When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and is frozen in a chamber at –20° or below as soon as possible and at the latest within 72 hours of collection. Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below –20°. For accidental reasons, the storage temperature may rise to above –20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds –20° does not exceed 72 hours; the temperature does not exceed –15° on more than one occasion; the temperature at no time exceeds –5°.
Plasma frozen within 24 hours after phlebotomy	
CFR	Plasma manufactured from whole blood should be frozen within 24 hours after phlebotomy. Blood component must be labeled "Plasma Frozen Within 24 Hours after Phlebotomy."

Appendix 2: Donor Criteria

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
General criteria for blood donation				
Appearance	Donor should appear to be in good health (AABB)	Donor should be in good health	Only donors in good health accepted	Nothing specific noted
Underlying medical conditions	Most serious medical conditions are grounds for deferral under United States, EU, and UK guidelines. FDA guidelines require deferral only if a person has used bovine insulin manufactured from UK cattle. However, AABB guidelines require deferral for cancer, heart, liver, or lung disease, and bleeding tendency unless approved by a medical director. Cancer and cardiac disease and diabetes treated with insulin require permanent deferral under EU and UK guidelines			Most serious medical conditions are grounds for deferral. Cancer: accepted 5 years of remission. Cardiac disease: varies depending on clinical condition. Diabetes: acceptable if controlled.
Age	≥16 or conform to applicable state law (AABB, whole blood)	Between 18 and 65; donation at 17 permitted if in accord with national legislation; first-time donors >60 only if permitted by physician	Whole blood: between 17 and 65; no first-time donors >60 Apheresis: first-time donors between 18 and 60; may donate up to age 65	Whole blood: can start at 16–17 with consent of parents and continue to 80, but medical review required at >70. Apheresis: accept new donors 18–65, with medical evaluation required when >60. Existing donors require annual medical review if >65.
Weight	None stated: no more than 10.5 mL/kg may be withdrawn (AABB) FDA requires Source Plasma donors to weigh at least 110 lb	Whole blood: ≥50 kg Apheresis: no specific weight requirement	Whole blood: ≥50 kg Apheresis: ≥50 kg	<45 kg—defer. Medical opinion required for unexplained weight loss
Blood pressure	Systolic ≤180 mm Hg Diastolic ≤100 mm Hg (AABB)	Systolic ≤180 mm Hg Diastolic ≤100 mm Hg	No specified blood pressure parameters; donors with high blood pressure may donate provided (1) they have not suffered any complications caused by high blood pressure, (2) they are taking only beta blockers and/or diuretics, and (3) their disease is stable, as determined by qualified medical personnel.	Acceptable ranges: Systolic 90–180 mm Hg. Diastolic 60–90 mm Hg. Hypertension 180–100: defer. Hypotension 90–60: defer.
Pulse	Between 50 and 100 beats per minute and regular; lower pulses acceptable at discretion of physician (AABB)	Between 50 and 100 beats per minute and regular	No specific pulse rate parameters stated	Regular pulse between 50 and 100: accept Pulse between 40 and 49: accept if donor is physically fit and not on medication
Temperature	≤37.5° taken orally at time of donation (AABB)	Donors who have had a temperature of ≥38° may not donate for 2 weeks	Donors who have had a temperature of ≥38° or flu-like symptoms may not donate for 2 weeks	No requirement

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Appendix 2: Donor Criteria *(continued)*

Criterion	Region			
	United States	United Kingdom	European Union	Australia
HgB/HCT	≥125 g/L or HCT 38%	Males: ≥135 g/L Females: ≥125 g/L	Males: ≥135 g/L Females: ≥125 g/L	Whole blood: females, 120–165 g/L; males, 130–185 g/L. Apheresis: females, 115–165 g/L; males, 125–185 g/L. If high, defer. If low, defer for 6 months and test for ferritin.
Skin examination	Free of infectious skin disease at site of phlebotomy; no skin punctures or scars indicative of addiction to self-injected narcotics (CFR) Free of infectious diseases (AABB)	Skin at venipuncture site should be clear of lesions, including eczema	There should be no skin disease at venipuncture site	Avoid venesection where there is evidence of inflammation or infection
Pregnancy	Defer for 6 weeks after delivery (AABB)	Defer 6 months after delivery	Defer for 1 week for every completed week of pregnancy	Current: defer 9 months from estimated date of confinement. After third-trimester delivery: defer 9 months. Miscarriage or termination: defer 3, 6, or 9 months, respectively for 1st, 2nd, and 3rd trimester.
Underlying medical conditions that require deferral and that do not pose a risk of transfusion-transmissible infection				
Cancer	Permanent deferral unless deemed suitable by medical director (AABB)	Permanent deferral although physician may make exceptions. Permitted after cervical cancer or basal cell carcinoma if successfully treated	Malignant neoplasms, including leukemias and myeloproliferative disorders, are cause for permanent deferral; exceptions may be made for certain conditions after successful therapy	Permanent deferral for haematological malignancies. Skin cancer–basal–cell carcinoma: accept. Other cancers: defer 5 years after completion of treatment
Cardiac disease	Free of major organ disease (heart, liver, and lungs) unless deemed suitable by medical director (AABB)	Permanent deferral for persons with a history of heart disease, especially coronary disease, angina pectoris, severe cardiac arrhythmia, arterial thrombosis, or recurrent venous thrombosis. 2-year deferral for rheumatic heart disease with no evidence of chronic disease	Permanent deferral for persons with active or past serious cardiovascular disease, except congenital abnormalities with complete cure	Permanent deferral for arrhythmias, endocarditis, ischaemic heart disease, heart surgery, myocardial disease. Accept: congenital heart disease if surgically corrected. Heart murmurs: accept, subject to medical opinion. Accept after full recovery: pericardial disease, rheumatic heart disease.
Cerebrovascular diseases	No specific guideline other than that the donor must be free of major organ disease (AABB)	Permanent deferral for history of cerebrovascular diseases	Permanent deferral for donors with a history of serious CNS disease	Permanent deferral
Epilepsy	No deferral	Must be free of epileptic attack for 3 years and have been taken off all medication	Permanent deferral unless at least 3 years have elapsed since the date that donor last took anticonvulsant medication and there has been no recurrence of symptoms	Defer for 2 years from last seizure
Gastrointestinal disease	No specific deferral; free of major organ disease unless deemed suitable by medical director (AABB)	No specific deferral	Deferral (not noted to be a permanent deferral) for disease that renders the individual liable to impaired iron absorption or blood loss	Ulcers: defer indefinitely
Genitourinary and renal disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Five-year deferral after complete recovery from acute glomerulonephritis	Permanent deferral for donor with serious genitourinary or renal disease	Permanent deferral: chronic pyelonephritis, chronic kidney infection, chronic dialysis. Accept if resolved: haematuria, acute kidney infection. Urinary catheter present: plasma only for fractionation if underlying condition acceptable. Acute dialysis: defer for 12 months. Acute glomerulonephritis: defer 5 years after recovery.

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Appendix 2: Donor Criteria *(continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Diabetes	No specific deferral except that receipt of bovine insulin manufactured in the UK requires permanent deferral (FDA). Free of major organ disease unless deemed suitable by medical director	Permanent deferral if insulin therapy required	Permanent deferral for donors on insulin treatment	Permanent deferral if diabetes-associated complications present Accept if disease is controlled, even if patient is taking insulin
Respiratory disease	Free of acute respiratory disease (CFR). Free of major organ disease (lungs) unless deemed suitable by medical director (AABB)	Permanent deferral for chronic bronchitis; common cold acceptable	Permanent deferral for serious disease	Permanent deferral: chronic abscess, bronchiectasis, or emphysema with respiratory insufficiency. Accept if mild and controlled: asthma, bronchiectasis without respiratory insufficiency Plasma for fractionation only: chronic bronchitis. Acute bronchitis: defer 2 weeks after recovery and being off antibiotics for 5 days. Pleurisy, pneumonia: defer 4 weeks after recovery. Acute pulmonary embolism: defer 12 months after recovery
Hematologic disorders	Free of abnormal bleeding tendency unless determined suitable by medical director (AABB) Free of major organ disease (cancer) unless deemed suitable by medical director (AABB)	No specific deferral. Donors who are heterozygous for beta thalassemia eligible if HgB values are within normal limits	Permanent deferral for donors with serious hematologic diseases	Permanent deferral: donors with serious hematologic diseases (sickle cell disease, thalassaemia major). Accept: thalassaemia minor. Defer: anaemia Plasma for fractionation only: elliptocytosis, glucose-6-phosphate dehydrogenase (G6PD) deficiency, spherocytosis. Apheresis only permitted for patients with G6PD deficiency
Immunologic disorders	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Permanent deferral for donors with autoimmune disease that affects more than one organ. Defer: documented history of anaphylaxis	Permanent deferral for donors with serious immunologic diseases	Permanent deferral: autoimmune disorders. Accept: If asymptomatic, only one organ system involved, not on immunosuppressive therapy. Accept: Sjogren's syndrome
Metabolic disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	No specific deferral	Permanent deferral for serious metabolic disease	No specific reference
Bone disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Two-year deferral after having been declared cured of osteomyelitis	No specific deferral	No specific reference
Surgery	No specific deferral unless blood was transfused; in which case, a 12-month deferral applies (CFR)	Major surgery requires evaluation of risk for transfusion-transmissible disease	Permanent deferral for persons with history of resection of the stomach. Major surgery requires a 6-month deferral; minor surgery requires a 1-week deferral	Minor (e.g., skin lesions, arthroscopy): defer until recovered. Routine minor (e.g., appendectomy, laparoscopy): defer for 2 months. Major surgery (if donor received autologous blood only): defer 6 months. Neurosurgery; medical assessment required.
Medications that require deferral				
Antibiotics	As defined by medical director (AABB)	Donors treated with any prescribed drug should be deferred for a period consistent with the pharmacokinetic properties of the drug	Defer for 2 weeks from full recovery or 1 week from cessation of antibiotic therapy, whichever is longer	Acute treatment: defer until recovered and off antibiotics for 5 days. Prophylactic: accept plasma only for fractionation. Topical: accept if skin is unbroken and not infected.

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Appendix 2: Donor Criteria *(continued)*

Criterion	Region			
	United States	United Kingdom	European Union	Australia
Drugs with teratogenic potential	Etretinate: permanent deferral (FDA). Acitretin: 3-year deferral from last dose (FDA). Dutasteride: 6-month deferral from last dose (FDA). Isotretinoin: 1-month deferral from last dose (FDA). Finasteride: 1-month deferral from last dose (FDA).	Donors treated with drugs with proven teratogenic effect should be deferred for a period consistent with the pharmacokinetic properties of the drug	Donors taking drugs that are proven or potential teratogens or who are taking drugs that accumulate in the tissues over long periods of time should not be used as blood donors	Raloxifene (Evista): defer for 6 months after completion of treatment. Acitretin (Neotigason): defer for 3 years after completion of treatment. Etretinate (Tigason): permanent deferral. Finasteride (Proscar): 7 days after completion of treatment. Isotretinoin: defer for 8 weeks after completion of treatment.
Growth hormone from human pituitary glands	Permanent deferral (FDA)			Permanent deferral
Other drugs	No other deferrals by FDA or AABB. Any other deferral at discretion of medical director of blood center (AABB). Warfarin: 7-day deferral for plasma donation (AABB).	Recommended that a list of commonly used drugs with rules for acceptability of donors, approved by the medical staff of the transfusion center, be available.	Other drugs acceptable as long as the underlying condition for which the drug is taken is acceptable.	Most medications that are taken by donors are not harmful to recipients; therefore people taking medications can be acceptable as blood donors. Eligibility is based on the assessment of the underlying condition and specific medication guidelines.
Insulin	Permanent deferral: bovine insulin made in UK (FDA).	Permanent deferral if treated with insulin	Permanent deferral if treated with insulin	Defer if diabetes is poorly controlled
Immunizations				
Toxoids	No deferral	No deferral	No deferral	
Licensed killed bacterial vaccines	No deferral	No deferral		No deferral
Licensed inactivated viral vaccines	No deferral	No deferral	No deferral except 1-week deferral after hepatitis B vaccination	No deferral except 1-week deferral after hepatitis B vaccination
Unlicensed killed vaccines	1-year deferral (AABB)			Defer 3 months after vaccination
Inactivated rickettsial vaccines	No deferral	No deferral		
Live attenuated bacterial and viral vaccines	4 weeks for varicella and rubella 2 weeks for rubeola, yellow fever, mumps, polio (oral), typhoid (oral) (AABB)	4 weeks	8 weeks	Plasma only for fractionation for 4 weeks after vaccination
Transfusion-transmissible infections that require deferral				
HIV infection/AIDS and sexual partners	Permanent deferral if present or past clinical or laboratory evidence of HIV infection/AIDS: positive EIA with positive or indeterminate confirmatory test; positive NAT test; clinical signs include unexplained weight loss, night sweats, blue or purple spots in mouth or on skin, white spots or unusual sores in the mouth, swollen lymph nodes for more than 1 month, persistent cough or shortness of breath, persistent diarrhea, fever for more than 10 days; sexual partners deferred for 1 year from time of last contact (FDA)	Permanent deferral for donors found to have a confirmed positive marker for HIV. Donors found to have a repeat positive marker for HIV that cannot be confirmed should be informed according to the nationally agreed algorithm.	Donors with HIV I or II must be permanently deferred	Infection: permanent deferral. Relevant symptoms within the last 6 months: defer for 12 months. Sexual contact with HIV-positive partner: defer for 12 months after last sexual contact.

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Appendix 2: Donor Criteria *(continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Hepatitis and sexual partners and household contacts	Permanent deferral for the following: History of viral hepatitis after the 11th birthday. Confirmed repeatedly reactive for HBsAg. Positive test for anti-HBc on more than 1 occasion (testing is not required for Source Plasma donors). Present or past laboratory or clinical evidence of infection with HCV. Sexual partners of patients with hepatitis deferred for 1 year from last contact; household contacts of persons with hepatitis B deferred for 1 year from last contact (CFR).	Permanent deferral for donors whose blood gives a positive reaction for the presence of HBsAg and/or anti-HCV. Donors with a history of jaundice or hepatitis may, at the discretion of the appropriate competent medical authority, be accepted as blood donors, provided that an approved test for HBsAg and anti-HCV is negative.	Permanent deferral for hepatitis B and C: donors with history of hepatitis B may donate after 12 months after recovery, provided that all markers are negative or core antibody positive, HBsAg is negative, and anti-HbS ≥100 IU/L; donors with documented current or past infection with hepatitis C are permanently deferred; donors with hepatitis A are deferred for 12 months.	Hepatitis B acute or past infection: defer for 12 months after recovery, then perform hepatitis testing. Hepatitis B chronic carrier: permanent deferral. Hepatitis B contact, sexual, mucosal, household: defer for 12 months from last exposure unless immune. Hepatitis B other contact: accept. Hepatitis C positive past infection: permanent deferral. Hepatitis C contact, sexual, mucosal, household: defer for 12 months from last exposure; other contact: accept.
HTLV	Present or past clinical or laboratory evidence of infection with HTLV I/II (positive EIA on 2 occasions). [HTLV not tested in Source Plasma donors] (FDA)	Permanent deferral for carriers of HTLV I/II	Permanent deferral for donors with HTLV I/II	Infection: permanent deferral. Repeat reactive status: plasma only for fractionation. Sexual contact: defer for 12 months after last contact. Household contact: accept.
West Nile virus	Donors with symptoms suspicious of or actual diagnosis of WNV deferred for 120 days. Donor testing positive on WNV NAT deferred for 120 days. Donor who develops symptoms of WNV within 2 weeks of donation should be deferred for 120 days. Donors implicated in possible transfusion-transmitted WNV infection should be deferred for 120 days (FDA).	Defer for 28 days after donor leaves an area with ongoing transmission to humans.	Defer for 6 months if donor was in area endemic for WNV and was diagnosed with or had symptoms consistent with WNV. Defer for 28 days after donor returns from endemic area, provided donor has no symptoms of WNV.	Infection: defer for 3 months after full recovery. Area exposure: plasma only for fractionation for 8 weeks after leaving risk area.
Chagas disease and babesiosis	Permanent deferral for history of Chagas disease; current FDA regulations do not require testing for Chagas disease. However, although not required, most facilities collecting blood for transfusion perform EIA test for Chagas and permanently defer following positive test. (AABB) FDA probably will not require antibody screening for fractionated or recovered plasma used for further manufacture. FDA has granted exemptions and permitted the collection and distribution of Source Plasma for further manufacture into noninjectable products from a donor known to have Chagas disease (CFR). Permanent deferral for history of babesiosis (AABB)	Permanent deferral for individuals with Chagas disease or history of Chagas disease; blood of persons who were born or have been transfused in areas where the disease is endemic should be used only for plasma fractionation products unless a validated test for infection is negative	Permanent deferral for individuals with Chagas disease. Individuals in the following categories may donate 6 months after leaving an endemic area, provided that a validated test for Chagas disease is negative (if a validated test is positive or not performed, the donor is permanently deferred): born in South or Central America, mother born in South or Central America, transfused in South or Central America; lived or worked in a rural subsistence community in South or Central America for 4 weeks or more	Infection: permanent deferral. Contact: accept. Chagas disease area resident: plasma only for fractionation, permanently. Chagas disease area visitor: plasma only for fractionation for 12 months after leaving endemic area

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Appendix 2: Donor Criteria *(continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Creutzfeld-Jakob disease (CJD) and variant CJD	Permanent deferral if donor: has diagnosis of CJD or nvCJD; has a blood relative diagnosed with CJD; received dura mater graft; received human-derived pituitary growth hormone; received bovine insulin made in UK; spent a cumulative 3 months in UK between 1980 and 1996; received a blood transfusion in the UK at any time since 1980; spent 6 months between 1980 and 1990 on a US military base in Northern Europe; spent 6 months between 1980 and 1996 on a US military base elsewhere in Europe; spent a cumulative 5 years in Europe (Source Plasma donors are not deferred for the latter) (FDA)	Permanent deferral if donor: treated with extracts derived from human pituitary glands; has been recipient of dura mater or corneal graft; has a family risk of CJD or any other TSE. For vCJD: Member states should determine on the basis of the prevalence of BSE within individual countries, of the endogenous exposure of the population to bovine products imported from countries with a high BSE prevalence, and of the incidence of cases of vCJD, what precautionary measures they may need to take to minimize the risk of transmission of vCJD via blood transfusion	Permanent deferral if donor: Diagnosed with CJD, vCJD, or any other prion-associated disease; has family risk of CJD; at increased risk from surgery, transfusion, or transplant of tissues or organs; received a dura mater graft; received a corneal, scleral, or ocular graft; received human-derived pituitary extract; received a blood transfusion in UK since 1980; received intravenous immunoglobulin (IVIg) of UK origin; donated unit of blood implicated in possible case of transfusion-related vCJD. Additionally, all plasma from British donors cannot be used for fractionation	Permanent deferral if diagnosed with any prion-related disease. Permanent deferral if donors have spent a cumulative time of 6 months in England, Wales, Scotland, Northern Ireland, the Channel Islands, or the Isle of Man between 1 January 1980 and 31 December 1996. Permanent deferral if donors received a blood transfusion or blood products in England, Wales, Scotland, Northern Ireland, the Channel Islands, or the Isle of Man from 1 January 1980 onwards unless the blood products were processed plasma products and were given after 31 December 2001. Permanent deferral if the donor had ear surgery performed between 1972 and 1989 and dura mater was used. Permanent deferral for donors who received human-derived pituitary growth and gonadotrophic fertility hormones prior to 1986.
Visceral leishmaniasis (Whole Blood)	Donors who have been to Iraq are deferred for 1 year. Permanent deferral for signs and symptoms of visceral leishmaniasis (FDA)	No specific deferral	Permanent deferral for visceral leishmaniasis	Cutaneous: plasma only for fractionation, permanently Visceral: permanent deferral Contact: accept.
Medical conditions and behaviors that place an individual at risk for a transfusion-transmissible infection and require deferral.				
Xenotransplant	No current deferral required for blood donation, but draft regulations for tissue and organs require permanent deferral.	Permanent deferral	Permanent deferral	Permanent deferral
Blood transfusion organ and tissue transplant; treatment with plasma-derived clotting factor concentrates	One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact	Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months	Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant	Blood transfusion homologous: defer 12 months Coagulation factor, blood derived, short term: defer 12 months from last treatment. Coagulation factor, blood derived, continuous: permanent deferral. Human tissue recipients: Organ/haematological: permanent deferral. Homologous, bone, tendon, skin: accept. Collagen: accept. Corneal: permanent deferral for iatrogenic cCJD risk.
Surgery or use of endoscope with biopsy	No specific deferral criteria; general health and transfusion criteria apply	Six-month deferral for major surgery; stomach resection requires permanent deferral; 1-week deferral for minor surgery; 6-month deferral for endoscope with biopsy; if NAT test for hepatitis C is negative, may donate after 4 months	Six-month deferral for major surgery or procedure using an endoscope; 1-week deferral for minor surgery	Defer 6 months
Nonsterile skin penetration or mucous membrane exposure to blood or body fluids not the donor's own	Twelve-month deferral	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months	Twelve-month deferral	Defer 12 months

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Appendix 2: Donor Criteria *(continued)*

Criterion	Region			
	United States	United Kingdom	European Union	Australia
Acupuncture, tattoo, body piercing, etc.	Twelve-month deferral unless performed by a state-regulated entity, using sterile needles and disposable dyes	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months; exception can be made according to national risk assessment	Twelve-month deferral; 6-month deferral if validated test for hepatitis B core antibody is negative; for acupuncture, no deferral if performed by state-regulated entity	If using single-use items: plasma only for fractionation for 12 months If not single-use or unsure: defer for 12 months
Injection of medications or steroids not prescribed by a physician	Permanent deferral; sexual partner deferred for 1 year	All blood donors should be provided with accurate and updated information about HIV transmission and AIDS so that persons who have unsafe sex practices or other risk behavior exposing them to potential infection will refrain from donating. The information provided may vary among countries according to local epidemiological data.	Permanent deferral if donor ever injected or has been injected with drugs; sexual partner deferred for 1 year	Permanent deferral
Males who have sexual contact with another male	Permanent deferral for sexual contact, even once, since 1977; female sexual partner deferred for 1 year from last contact		Permanent deferral for oral or anal sexual contact even if protection used; female sexual partner deferred for 1 year from last contact.	Defer for 12 months after last sexual contact
Accepted money or drugs or other payment in exchange for sex since 1977	Permanent deferral; sexual partner deferred for 1 year from last contact		Permanent deferral; sexual partner deferred for 1 year from last contact.	Defer for 12 months after last sexual contact
Incarceration for more than 72 hours in the past year	One-year deferral		No deferral	Defer for 12 months after release
Born in or lived in Africa	Permanent deferral if born or lived in countries where HIV I subtype O is endemic (Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, Gabon, Niger, Nigeria); sexual partner deferred for 1 year from last contact unless tested with a test validated to detect Group O		No specific deferral for Africa; malaria rules apply; however, sexual partners of persons who were sexually active in areas where HIV is endemic deferred for 1 year from last contact	No specific deferral: donors who have visited a malaria-endemic area are subject to a plasma-only restriction period of at least 12 months. The restriction period is extended to 3 years if residence has been for 6 continuous months or more within the past 3 years. With a negative malaria test, the restriction period can be reduced to 4 months. Donors who have traveled to an HIV risk area must be asked if they had sexual contact with a resident of that area.

Appendix 3: Disease Testing

Disease Test	United States	European Union
Hepatitis		
HBsAg	Permanent deferral for repeatedly reactive test results (FDA)	***European Pharmacopoeia:* Human Plasma for Fractionation**
Hepatitis B core antibody	Permanent deferral if reactive on 2 or more separate occasions; permanent deferral if core antibody positive results are coupled with prior or concurrent repeatedly reactive HBsAg test (FDA). Testing is not required for Source Plasma donors	Laboratory tests are carried out for each donation to detect the following viral markers: (1) antibodies against HIV-1 (anti-HIV-1). (2) antibodies against HIV-2 (anti-HIV-2). (3) hepatitis B surface antigen (HBsAg). (4) antibodies against hepatitis C virus (anti-HCV). If a repeat-reactive result is found in any of these tests, the donation is not accepted.
Hepatitis C antibody EIA	Permanent deferral if repeatedly reactive for hepatitis C antibody; may re-enter donor after 6 months if confirmatory test is negative (FDA)	**Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations**
Hepatitis C NAT testing	Permanent deferral if positive on single testing (FDA)	The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations:

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Appendix 3: Disease Testing *(continued)*

Disease Test	United States	European Union
		ABO group (not required for plasma intended only for fractionation). Rh D group (not required for plasma intended only for fractionation). Testing for the following infections is required in donors: Hepatitis B (HBsAg), Hepatitis C (Anti-HCV), HIV 1 and 2 (Anti-HIV 1 and 2). Additional tests may be required for specific components or donors or epidemiological situations. **Blood Directive 2004/33/EC (Annex III): Permanent Deferral Criteria (excerpt):** Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune Hepatitis C HIV 1 or 2
HIV		
HIV NAT	Permanent deferral if positive on single testing (FDA)	
HIV I or II Antibody (EIA)	Permanent deferral for repeatedly reactive HIV EIA test; may reenter after 6 months if confirmatory test negative (FDA). Testing is not required for Source Plasma donors	*European Pharmacopoeia:* **Human Plasma for Fractionation** Laboratory tests are carried out for each donation to detect the following viral markers: (1) antibodies against HIV-1 (anti-HIV-1) (2) antibodies against HIV-2 (anti-HIV-2) (3) hepatitis B surface antigen (HBsAg) (4) antibodies against hepatitis C virus (anti-HCV) If a repeat-reactive result is found in any of these tests, the donation is not accepted **Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations** The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations: ABO group (not required for plasma intended only for fractionation) Rh D group (not required for plasma intended only for fractionation) Testing for the following infections are required in donors: Hepatitis B (HBsAg) Hepatitis C (Anti-HCV) HIV 1 or 2 (Anti-HIV 1 and 2) Additional tests may be required for specific components or donors or epidemiological situations. **Blood Directive 2004/33/EC (Annex III), Permanent Deferral Criteria (excerpt)** Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune Hepatitis C HIV 1 or 2

⟨1181⟩ SCANNING ELECTRON MICROSCOPY

INTRODUCTION

Over the last few decades, electron microscopy has become a reliable investigative tool for the study of solid and semi-solid materials. Since the invention of the electron microscope attributed to Max Knoll in 1935 and commercialization in the 1960's by Cambridge Instrument Co. and JEOL, recent improvements in resolution, stability, and specimen accommodation have resulted in a robust array of commercial instrumentation that spans a wide range of capabilities. This chapter provides a review of the common electron microscopy technologies and techniques.

UTILITY OF THE TECHNIQUE

General Description of the Technique

Scanning electron microscopy (SEM) is an electron optical imaging technique that yields both topographic images and elemental information and is used in conjunction with a variety of detection systems and elemental detectors. All electron microscopy systems utilize a high energy, focused electron beam as source illumination with concurrent increase in resolution compared to photon (light) microscopy. The resolution increase is realized by electron beam wavelength being three orders of magnitude smaller than that of visible light. The optics consist of condenser and objective lenses in conjunction with selected

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apertures. A focused e-beam is rastered over the surface of a specimen, generating a variety of signals from which an image is collected.

Traditional High Vacuum SEM

Traditional SEM systems operate at high vacuum, so specimens must be clean, dry, and able to withstand imaging at high vacuum, up to 10^{-6} torr. Additionally, the specimens must be inherently conductive or coated to conduct the high surface charges. Four general principles of these systems are:

- e-beam generation
- image formation
- specimen preparation
- elemental composition.

Typically, SEM analysis requires only a small amount (10^{-3} to 10^{-12} g, depending on the application) of a solid specimen. The specimen is presented to the collimated e-beam and scanned in a controlled raster pattern. Images, or micrographs, are generated using secondary electron (SE) and/or back-scattered electron (BSE) detection. Specimen detail may be resolved to 10 nm even in the most simple systems. The collimation of the e-beam also yields great depth of field that is evident in a wide dynamic range of magnification, practically spanning five orders of magnitude (5×–100,000×).

The size of the final aperture controls the beam diameter and, accordingly, the image resolution and total current at a specimen. Small apertures are required for high resolution and large apertures provide high current for optimal X-ray emission intensity. In many systems, the objective aperture can be adjusted during use with a sliding or rotating holder. Flexibility in trading resolution for specimen current is also important because specimen characteristics affect these two criteria differently.

Image magnification is controlled by altering the area of the electron beam raster; smaller areas yield higher magnification. An Everhart-Thornley (ET) detector is used for electron detection; the resultant images are most similar to those of reflected light microscopy. An ET detector consists of a Faraday cage and a scintillator disk connected by a light pipe to a photomultiplier tube. The Faraday cage serves three functions: (1) at positive bias it attracts SE; (2) at negative bias it repels SE to enable the ET detector to collect BSE signals alone; and (3) it shields the primary electron (PE) beam from the scintillator potential. Various scintillator coatings are used. For example, phosphorus based coatings yield intense, high-contrast images. Aluminum-based coatings, although less sensitive, can withstand the high SE flux generated during elemental analyses. Additionally, solid-state detectors provide up to 10 times greater sensitivity for BSE collection. They can be placed at a variety of positions and distances with respect to a specimen and used in conjunction with ET detectors.

Variable Pressure or Environmental Scanning Electron Microscopy (ESEM)

Robust and common variations of the traditional high vacuum system prevail in today's labs. So-called variable pressure, "environmental" or low vacuum systems (LVSEM) appeared in the mid 1980s. These are electron microscope systems operating at a much higher, near ambient pressure state (up to 50 torr) at the specimen site, plus gas over-blanketing (almost any may be used) and temperature control to 1500°C as well.

There are two key advantages for ESEM's: First, non-conducting samples do not require a conductive carbon or metal thin film to be applied. The gas in the sample chamber is ionized by the electrons generated from the beam/sample interaction. These ions will diffuse to the sample surface, thereby dissipating any negative charge buildup. Secondly, if the sample in question is prone to outgassing volatile substances such as solvents and water, the near-ambient pressures used in the sample chamber can greatly diminish the likelihood of this occurring.

Scanning Transmission Electron Microscopy

The combination of a traditional transmission electron microscope system with scanning control yields specimen analysis with high micrograph resolution, information at the atomic scale such as atomic structure, chemical information, and interfacial bonding. Image acquisition is conducted in the same manner as SEM; however, use of thin specimens allows collection of transmission images as well. Great increase in resolution is attained by controlling the e-beam probe volume, to atomic dimensions. *Figure 1* shows the lattice image of a ceramic that was obtained using a scanning transmission electron microscopy (STEM) instrument. As with any electron- or ion-beam method, the sample must be able to withstand the electron bombardment. This may not always be the case with pharmaceutical materials, as they can degrade in the e-beam during the analysis.

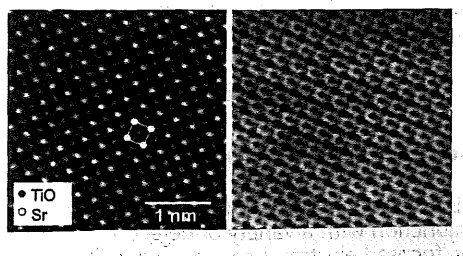

Figure 1. Scanning transmission electron micrograph of an oxide ceramic (perovskite), shown in both dark field (left) and bright field (right) imaging modes. Image courtesy of "... © Carl Hanser Verlag, Muenchen".

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ELECTRON BEAM GENERATION

The basis for the formation of an electron image is the generation of an energetic flux of electrons that impinge upon the sample surface. If the beam is too energetic, the sample will be burned as the beam is rastered across the sample's surface. This is an important consideration when analyzing pharmaceutical materials.

Electron guns can be classified into two types: thermionic emitters and field emitters. With the former, the filament is heated under high vacuum, and a large potential is applied. The field that is created, combined with the heated filament, "boils" off electrons that are subsequently focused in the microscope optics. To increase the flux of electrons from thermionic emitters, one must increase the potential that is applied across the filament, which also increases the kinetic energy of the electrons and the likelihood that the sample surface will be burned.

Another method used to produce electronic beams is to apply a large electric field across an electronic emitter. The filament can have a current passing through it or not. Where surface damage is a prime consideration, cold emitters are the best way to obtain a clear image at high magnification without burning the surface or damaging the material. Typically tungsten is used as the electron emitter, as it is strong enough to withstand the mechanical forces. When using a field emission gun, the cathode must be kept at very high vacuum, and no foreign contaminants must be allowed to deposit on the filament. For these reasons, the column must be differentially pumped. Field emission instruments are better suited for the analysis of pharmaceutical compounds, as they produce a very bright beam that is less damaging to the sample. Additionally, field emission electron SEMs have higher spatial resolution, making them better suited for the identification of foreign particulate matter.

Electron Beam–Sample Interactions

Interaction of the electron beam (PE) with the specimen produces a variety of physical phenomena that, when detected, are used to form images and provide elemental information about the specimen. These phenomena include:

- emission of inelastic scattered secondary electrons (SE), proportional to specimen topography
- reflection of elastically scattered back-scattered electrons (BSE), proportional to the atomic number (*Z*) of the specimen
- component X-ray emission (XRE, X in *Figure 2*)
- emission of Auger electrons (AE)
- cathodoluminescence (CL)
- conduction of current (specimen current)
- charging from induced voltages (IV) or adsorbed electrons
- electron transmission
- heat generation
- electromotive forces (see *Figure 2*).

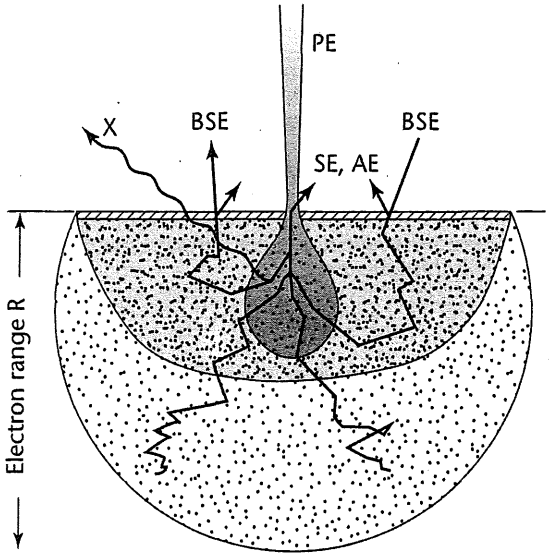

Figure 2. Interaction diagram.

SE are emitted from a specimen surface as the result of inelastic collisions between primary (incident) electrons and the specimen. When the energy imparted to a specimen electron exceeds the work function of a specimen, that electron is emitted as SE. Most secondary electrons have energies of 5–20 eV; electrons in this low-energy range can be efficiently collected, yielding high signal-to-noise images. Because such low-energy electrons can penetrate only short distances through the specimen, SE originate from within 2–30 nm of the surface and generate highly resolved images. The actual e-beam penetration depth is dependent on e-beam accelerating voltage, specimen elemental composition, specimen density, and specimen mounting angle. Excitation volumes of 0.5–5 µm in diameter (dark gray) are typical for a wide range of materials from metallic to biological.

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Back-scattered electrons are primaries that have been reflected from the specimen. The primary electrons can undergo multiple collisions prior to exiting from the specimen; therefore, BSE have energies over a broad range and emerge from relatively deep specimen penetration (≈0.1–5 µm) (see *Figure 3*).

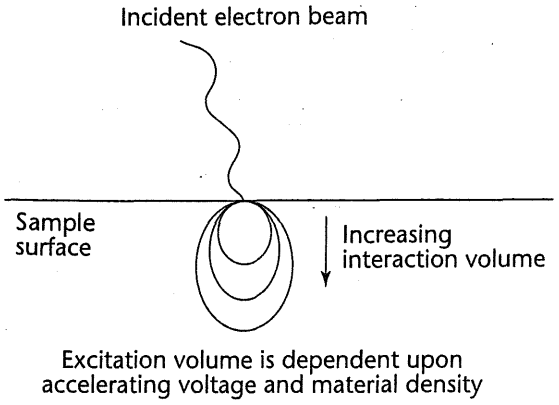

Figure 3. Bulk penetration.

These high-energy (15–25 keV) BSE are collected less efficiently than SE, and they yield images with poorer resolution. The efficiency of BSE reflection is a function of the atomic number (*Z*) of the specimen atoms; thus, the contrast of BSE images depends on elemental composition. The penetration depth of all electrons is affected by elemental composition, specimen density, specimen tilt, and incident beam energy (accelerating voltage). There are a number of software algorithms to correct for these influences, when known such as ZAF, Bence-Albee, etc. For example, the SE images of sodium phosphate and zinc phosphate crystals are quite similar. However, the heavier nuclei of the zinc species produce more efficient BSE reflection, and BSE images will reveal higher contrast. BSE images of heavy- versus light-element phases, or mixtures of species, show dramatic contrast differences that are representative of elemental heterogeneity. Elemental mapping by energy dispersive X-ray spectroscopy (EDS), discussed later, can provide complementary information.

SAMPLE PREPARATION

As scanning electron microscopy has advanced as an analytical technique, different options have become available for sample preparation. Ideally, the objective is to obtain the required image or chemical analysis of the material to be examined with as little intervention as possible. Additionally, one must consider the purpose of the analysis and the amount of material that is available. If the material to be examined is a foreign particulate in a parenteral product, sample preparation could be quite different than if one is intending to image a bulk drug substance material or excipients. The decision tree (see *Figure 4*) below represents a simplified version of the available sample preparation method and is one possible construct to determine the appropriate sample preparation method.

For example, in addition to metal (primarily aluminum) sample stubs there are also various metal meshes that can be used to support samples. If the particle in question is isolated from liquid, the filter itself may serve as a suitable substrate for the powder without having to transfer the sample to another sample holder. Additionally, adhesive carbon tabs are a common means to secure the sample to the stub. There are a variety of other adhesive materials such as double-sided copper, silver and aluminum tape, or carbon and silver paint for this purpose.

Sample Coating

Historically, pharmaceutical materials, which are typically poor electrical conductors, had to be coated with a very thin layer of conductive material (carbon, gold, platinum, or other metals) to ensure that the electron beam used for imaging could be dissipated from the sample surface. Coating was also used to create a layer of "electron rich" material that would serve as a source for secondary electron emission.

With the advent of environmental scanning electron microscopy (ESEM) or variable pressure scanning electron microscopes, coating of non-conductive materials is no longer a prerequisite. The atmosphere in the sample chamber itself generates positively charged ions that will dissipate any charge that accumulates on the sample surface.

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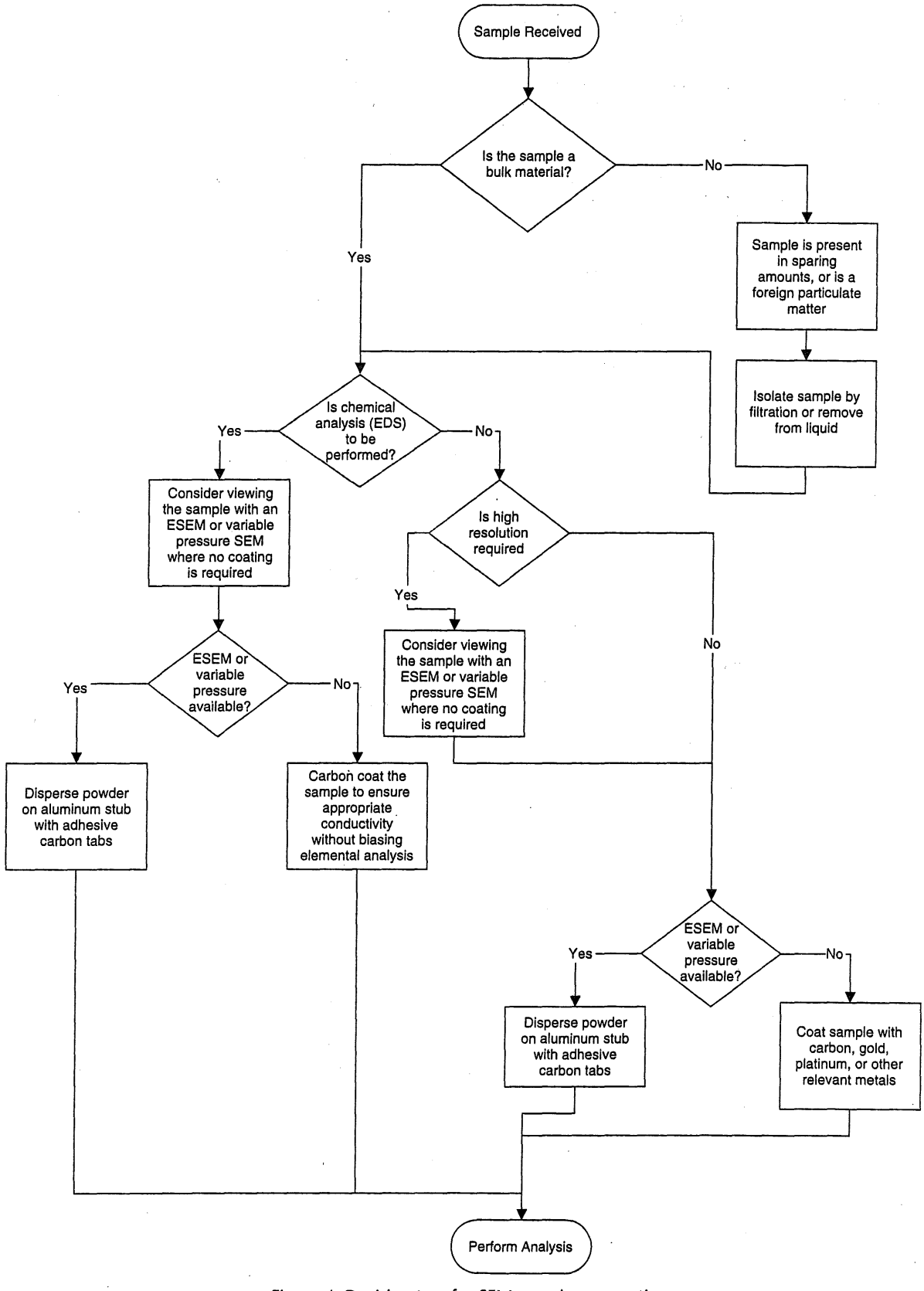

Figure 4. Decision tree for SEM sample preparation.

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X-RAY GENERATION AND ELEMENTAL COMPOSITIONAL ANALYSIS

When a PE encounters an orbital electron in an atom, the resultant collision can either promote that orbital electron to a higher energy level or ionize the atom. Stabilization of an atom by relaxation of a higher energy electron to fill a vacancy results in the emission of an X-ray photon. These X-ray energies are discrete and element-specific; they equal the differences between the shell electron energies for the various shells of a given element. For instance, an ejected K-shell electron can be stabilized by a higher energy L-shell electron, yielding a net energy ($E_L - E_K$), which is specific for the X-ray photon energy of the elemental K line. X-ray emission lines are classified according to the electron shell in which the vacancy existed, e.g., K, L, M. The lines are further categorized according to the shell from which the relaxing electron originates. Thus, a Kα X-ray line will arise from a vacancy in a K-shell that is filled from an L-shell; a Kβ X-ray line will arise from a K-shell vacancy filled from an M-shell, and so on (see *Figure 5*).

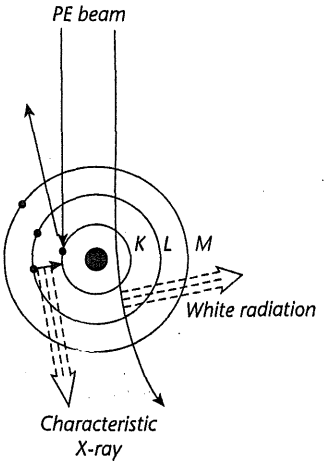

Figure 5. Atom model.

Detection of the generated X-rays and subsequent elemental analysis of the feature of interest can be accomplished by two different kinds of detectors: wavelength and energy dispersive X-ray spectrometers (WDS and EDS, respectively). Each has its individual benefits and challenges. Historically, wavelength dispersive detectors are much less susceptible to spectral artifacts that can create incorrect peak identification such as peak broadening and distortion, silicon escape peaks, absorption, etc. Additionally, most Si(Li) EDS detectors require liquid nitrogen cooling, which can cause ice build-up on the detector window in the sample chamber. The much larger EDS collection angle makes it more robust and less susceptible to detector misalignment than WDS. *Figure 6* illustrates the superior spectral resolution that is obtained with WDS. With WDS, it is much easier to detect characteristic X-rays that are energetically similar. The EDS exhibits peak broadening due to the close proximity of the peaks.

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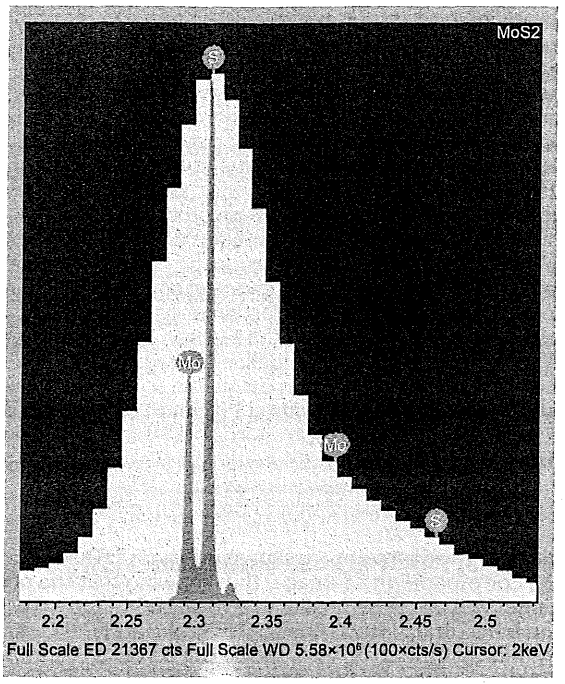

Figure 6. Example of spectral resolution obtained with wavelength-dispersive detectors where S Kα and Mo Lα can be clearly resolved by WDS (gray peaks) but are not resolved by EDS (broad yellow peak). "Used by Permission. Courtesy of Oxford Instruments."

Advantages of EDS reside in the ease of use and speed to collect the entire X-ray spectrum making it ideal for the routine identification of particles and features within a sample. The two types of detector technology most widely available for energy dispersive X-ray spectroscopy (EDS) analysis are based on lithium-drifted silicon [Si(Li)] and silicon drift technology. While Si(Li) detectors have been around for the last 40 years, silicon drift detectors (SDD) have recently emerged as the preferred type due to several key advantages including better detection of light elements ($Z \geq 4$), ability to handle high count rates, excellent energy resolution even at high count rates, and even elimination of the need for liquid nitrogen to cool the detector prior to use due to the use of Peltier cooling systems. While Si(Li) detectors were generally recommended to be run at 1,000–2,500 counts per second with an energy resolution of 129 eV at Mn-Kα, current SDD technology has superior energy resolution of 121 eV even at the high count rate of 600,000 cps. For the average user, this means that the SDD detector may drastically shorten analysis time provided that a high count rate may be obtained and with significantly better resolution for detection of light elements. In practicality, the achievable count rate is determined by the accelerating voltage and probe current, solid angle of the detector and working distance to the sample, and ability of the sample to survive the electron beam without charging or material change. Whether the user has an Si(Li) or SDD detector, it is a good rule of thumb to keep the dead time to 30% or below.

The elemental content of a specimen has a bearing on the selection of conditions for analysis. The most useful range of accelerating voltage is approximately 3–20 kV; most elements of interest can be ionized by electrons with energies in this range. The energy required in order to excite X-ray emission from a given line is termed its critical excitation potential. The critical excitation potential for a K line can be approximated by the sum of the primary line energies (Kα + Lα + Mα). Selection of an accelerating voltage equal to 1.5 times this sum is usually sufficient for semi-quantitative analysis. For example, copper has Kα at 8.05 keV + Lα at 0.93 keV = 8.98 keV: and 1.5 × 8.98 keV = 13.47 keV. Selection of 15-kV accelerating voltage yields sufficient energy to ionize the K-shell of copper atoms and generate a useful analytical signal. It is generally suggested that EDS practitioners should use the minimum accelerating voltage necessary to adequately excite the elements of interest (typically 1.5–10 times the critical excitation energy). The interaction volume increases with increasing accelerating voltage, thus better spatial resolution can be achieved by using lower accelerating voltage. Depending on the dimensions and density of the particles, a signal from the substrate may be detected. Additionally, larger interaction volume causes increased absorbance and fluorescence within the sample volume. Several accelerating voltages may be experimented with to determine the minimum accelerating voltage required. Routine use of a copper and a lighter metal standard such as aluminum allows quick verification of elemental primary line energies bracketing the 0- to 10-keV range. An elemental collection for the metal standard prior to or within the experimental protocol is conducted to verify the primary line is ±0.04 keV of reference (but may depend upon the detector manufacturer specification). This is a practical internal standard practice.

Inter-element interferences originate from many effects. High-energy X-rays emitted from heavy atoms can ionize lighter elements to produce secondary X-ray emission from the lighter species. Lower high-Z element fluorescence and higher low-Z element fluorescence can be observed, in contrast to that expected from the PE-induced signal of a pure element. Conversely, X-ray emission from a light element may be absorbed by a heavier matrix to yield a negative bias in the light-element signal. These effects always exist in heterogeneous specimens and must be corrected for during any quantitative analysis. A common algorithm, ZAF, may be used to correct for Z-dependent interferences due to absorption and secondary X-ray emission.

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Point analysis and mapping are common analysis modes available for the identification of particles by EDS regardless of detector type. Most modern user interfaces allow the user to view a region of interest with secondary electron imaging and collect a spectrum for the entire region or a defined sub-region. Upon collecting an initial spectrum, it is important the user check that it appears satisfactory in terms of energy range for the elements present, optimum count rate, and is free from artifacts such as charging. Once optimized for collection, the user can choose their preferred analysis mode to identify their sample. Point analysis is generally useful if different particle morphologies can be readily observed by SEM. The user can place a cross-hair at the center of each particle to collect a spectrum for later comparison. Care should be taken for the analysis of particles less than 5 µm since the interaction volume may spread in the sample combined with the beam/sample interaction and can excite electrons from the surrounding environment. This will produce spectral peaks that are not native to the feature of interest. For this reason, chemical analysis of small objects in an SEM is difficult. Other more surface-sensitive techniques such as X-ray photoelectron spectroscopy (XPS), scanning auger spectroscopy, and others provide more accurate spectral information. Orthogonal analysis, both within and outside any given technique, is of great value.

When there is no obvious morphological difference for a collection of particles, EDS mapping may be the best mode of analysis. In this mode, the EDS detector collects information as the electron beam rasters across the sample, thus the elemental information is in registry with the SE/BSE image. The user can define the region of interest from the entire region observable in the SE/BSE image to a sub-region. Additionally, the user can define the scan speed, dwell time/pixel, and resolution of the map to suit their needs in terms of speed or resolution. A significant factor to consider for EDS mapping is that the X-ray signal will vary based on the surface texture and slope of the particle's surface. In the most severe case, a shadow may be observed if that surface is no longer within the line of sight of the detector. It may be possible to minimize these artifacts due to tilting the sample further towards the detector. It is often useful to map the elements of interest versus an element that does not exist in the sample. The X-ray counts for the non-existent element should provide a uniform background across the entire region if the surface is level.

Examples of elemental maps overlaid on the SE image are shown in *Figure 7*. The first image (*Figure 7*, left) shows maps for barium and potassium overlaid on the corresponding SE image, thus showing that the particles represent an inhomogeneous mixture of barium/potassium precipitates (left). For the second example, EDS mapping was used with Cryo-SEM to examine a cryo-fractured silicone/water emulsion where the Si map represents silicone and the O map represents water. The internal structure of a large silicone droplet within a continuous water phase can be observed in *Figure 7* (right) where it is apparent the silicone droplet contains several internal water droplets. Many current EDS interfaces allow the user to extract a spectrum from any location within the map.

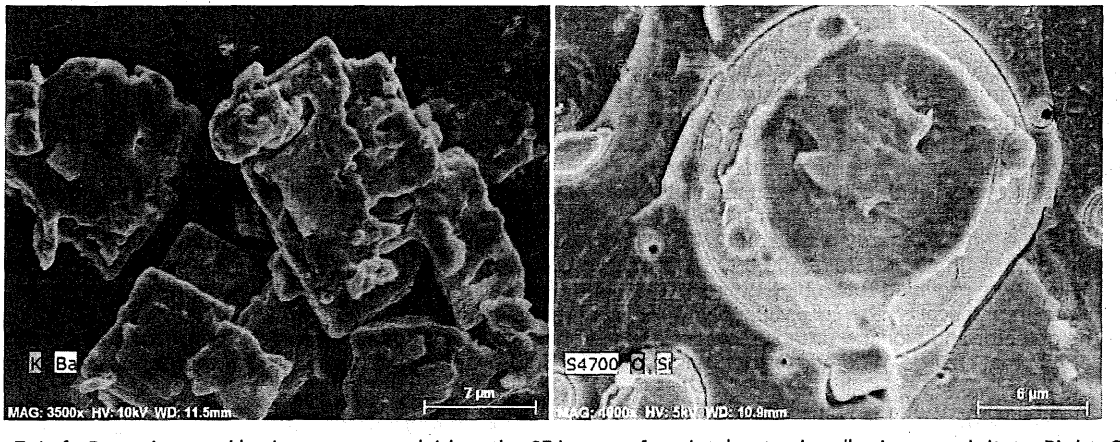

Figure 7. Left: Potassium and barium maps overlaid on the SE image of a mixed potassium/barium precipitate. Right: Oxygen and silicon maps overlaid on SE image of a vitrified silicone/water emulsion.

IMAGE OPTIMIZATION

Instrumental parameters needed to acquire a quality image can be highly dependent on sample type and sample preparation. Some important aspects with regard to the sample include the form (powders, monoliths, fibers, vitrified liquids, etc.), overall dimensions, texture, atomic composition, and inherent conductivity. Since many instrumental parameters for a modern SEM can be readily modified with the PC-based user interface with only slight re-optimization, it is highly suggested that the operator experiment with operating conditions in order to choose the best imaging conditions for the features of interest. Parameters that affect image quality can be further categorized as instrumental settings and observation settings.

Common instrumental settings that may affect image quality include accelerating voltage, emission current, working distance, SE/BSE detectors (if multiple modes are used), condenser lens current setting, and objective lens aperture diameter. Of these, the accelerating voltage may be the most important instrumental parameter since it directly affects the penetration depth of incident electrons thereby determining the features that can be observed. In general, choosing the optimal accelerating voltage is a balance of achieving the best contrast for the features that the user is most interested in, while minimizing unwanted effects such as loss of topographic information or charging. The best approach to choosing an ideal accelerating voltage for an unknown sample is to experiment with up to 3 accelerating voltages (e.g., 1, 3, and 10 kV), then choose the best one to continue to further optimize the image. The working distance and choice of SE/BSE detector are normally chosen in combination depending on the desired resolution, depth of focus, and sample tilt. Generally, shorter working distances result in higher resolution but less depth of focus. Likewise, based on the position the SE detectors are placed in the column, a tradeoff exists

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for spatial resolution versus surface topography, where upper detectors generally detect a high resolution SE signal and lower detectors show more surface topography. Lastly, the condenser lens setting and objective lens aperture diameter can be optimized to provide the ideal specimen current, resolution, and depth of focus required for a specific sample type.

Upon choosing an initial set of instrumental conditions as mentioned above, basic image observation can be optimized in the following manner:

1. Select a low magnification (1000–3000×) initially
2. Course adjust image brightness
3. Course adjust focus
4. Select an easy-to-visualize area of the specimen
5. Check/adjust to optimize the electromagnetic alignment of the electron optics (beam, aperture, stigmation)
6. Select a feature of interest at higher magnification
7. Adjust focus and astigmatism
8. Adjust image brightness and contrast
9. Collect image.

Image collection can be accomplished using different resolutions (1280 × 960 is most common) and capture speeds using either slow scan collection or frame integration. In general, slow scan collection is used to collect high-resolution images whereas frame integration is used for specimen types that have charging or stability issues. A collection using a higher capture resolution, slower scan speed, or additional frames may improve the image resolution, however the user must take caution of the increased likelihood of specimen drift over this period which would negate any benefit.

FORENSIC ANALYSIS OF PARTICLES

Shape Analysis

Because of the three-dimensional nature of images obtained via scanning electron microscopy, particle shape provides a great deal of information about the composition and source of the particle without obtaining a chemical analysis via EDS.

The most commonly identified particles found in pharmaceutical compounds generally fall into several distinct categories. Note that particles in parenteral products arise from three general sources: (a) extrinsic or outside of the product, as truly foreign solids; (b) intrinsic or associated with the product, its package or manufacture; (c) inherent particles expected to compose the formulation, such as proteins in biotherapeutic products (see *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩), and may be encountered when probing those formulae. Some examples of extrinsic particles are the following:

- Corrosion products from one or more equipment sources, and may occur from formulation interaction
- Materials from the manufacturing environment such as cellulose (from paper bags), extraneous fibers (clothing, filters, etc.)
- Inhomogeneities in the product, specifically incomplete mixing (for solid oral dosage products) that can create cosmetic or elegance issues with tablets
- Environmentally-sourced biological materials
- Material that has been exposed to extreme heat that might char. The causes for this can vary from paper pyrolized during depyrogenation to material sticking to components that are exposed to friction (powder milling, compaction, etc.). If residue unintentionally builds up in these areas, the exposure to friction can decompose the material, making it appear burnt.

Figure 8 shows representative images of some of the particulate matter mentioned above that the user might encounter in a forensic investigation.

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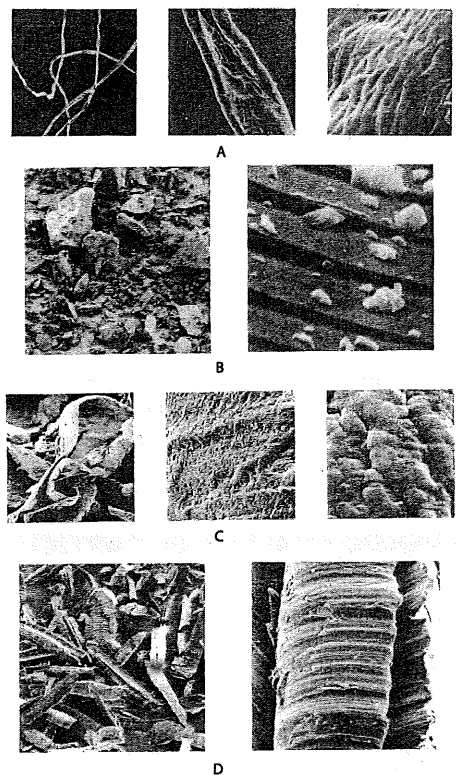

Figure 8. Scanning electron micrographs of commonly found particles in pharmaceutical manufacturing areas: A. cotton fibers, B. glass particles, C. Teflon shavings, D. aluminum shavings. (All of the images provided courtesy of McCrone Atlas (www.mccroneatlas.com.)

Interpretation of a particle's origin from characteristics observed by SEM analysis depends on the particle features; for instance, any particle with sharp corners and well-defined edges is made from a material that is not soluble in the product liquid. If it is transparent to the beam, then the user could conclude it consists of very low Z elements. Crystalline particles exhibit obvious symmetry and growth patterns. Slow growth is characterized by large crystals and fast growth by small, often aggregated crystal masses.

Particle morphology and surface characteristics may be helpful in sourcing unknowns. Aspect ratio, or the ratio of the length of a particle divided by its width, indicates fibrous materials. Analysis by optical microscopy often serves as a more effective tool to analyze fibers by determining diagnostic optical properties (refractive indices, birefringence, elongation, etc.) using plane and crossed polarized light that can distinguish among various possible sources. With a scanning electron microscope, however, elemental analysis can determine (a) if the fiber is inorganic or organic and (b) the presence of surface additives. Scanning electron microscopy also offers the added benefit of being able to image at much higher magnification to reveal morphological structures of biological origin or features indicative of commercial manufacture.

As mentioned previously, different kinds of electrons are emitted when an electron beam interacts with a solid surface. The two most useful types of electrons are secondary and back-scattered. By choosing the appropriate detector or instrument set up, the image can be biased towards one or the other. Back-scattered electrons can provide useful information regarding the chemical composition of the feature in question. If the instrument is set up to use BSE to form the image, elements possessing higher atomic mass will appear brighter than those having a lower atomic mass. By selecting this imaging mode, organic particles are easily distinguished from metallic debris, for example. For instance, at low kV, glass or plastic may appear as solid objects, whereas at higher kV they may appear somewhat transparent. Further, appearance at low kV on a tungsten filament SEM may mean something completely different than that at low kV on an FE SEM or environmental SEM. Different materials require different operating conditions to suitably image using SE, to prevent charging and to improve contrast. Typically, use low kV (3 kV or less using a semi-in-lens or in-lens FE SEM) for non-conductive materials such as glass and plastic. If the material is extremely thin (<100 nm), the material can be visualized in transmission mode (STEM) as well as SE mode.

GENERAL REFERENCES

1. Goldstein, J., et al., *Scanning Electron Microscopy and X-ray Microanalysis*. Third ed. 2003, New York: Springer.
2. Echlin, P., *Handbook of Sample Preparation for Scanning Electron Microscopy and X-ray Microanalysis*. 2010, New York: Springer. 330.
3. Reimer, L., *Scanning Electron Microscopy: Physics of Image Formation and Microanalysis*. Springer Series in Optical Sciences, ed. H.K.V. Lotsch. Vol. 45. 1998, New York: Springer.

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⟨1184⟩ SENSITIZATION TESTING

INTRODUCTION

This chapter considers sensitization and hypersensitization in the context of medical devices and implants, and describes methodologies for testing such articles for their potential to cause sensitization.

There are four types of hypersensitization reactions according to the Gell and Coombs classification system. Type I reactions involve the fixation of IgE to mast cells that subsequently release pharmacologically active substances, such as histamine. Type II reactions are the result of IgG and/or IgM binding to target cells, followed by complement fixation and cell lysis. Type III reactions are caused by the presence of antigen-antibody complexes that cause physical damage such as kidney damage due to glomerular blockage. Type IV reactions are cell-mediated (involve the action of T cells and their interaction with the human lymphocyte antigens). Type IV reactions are also called delayed-type hypersensitivity reactions. *Table 1* below summarizes the types of reactions, the mediators of the reactions, and examples of representative diseases.

Table 1. The Four Types of Hypersensitization Reactions*, Mediators, and Disease Examples

Reaction Class	Mediators	Disease Examples
Type I	IgE molecules bound to mast cells interact with antigen to release pharmacologically active substances	Hay fever, bronchial asthma, other atopic reactions
Type II	IgM and/or IgM molecules interact with target cells, fix complement, cell lysis	Various drug allergies, erythroblastosis fetalis, hemolytic anemia, thrombocytopenia
Type III	Antigen-antibody complexes, complement	Arthus reaction, serum sickness, allergic glomerulonephritis
Type IV	T lymphocytes, antigen, monocytes, macrophages	Contact dermatitis

* According to Gell and Coombs classification scheme

A multi-step process, delineated in chapter *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩ is followed in determining which, if any, toxicological tests need to be performed on a given article. In some cases, sufficient evidence to satisfy toxicology requirements may be available from previously marketed articles (See *Figure 1* in chapter ⟨1031⟩). Important factors addressed in *Figure 1* (chapter ⟨1031⟩) include the type and extent of contact with the body, the chemical composition, the manufacturing process, the sterilization process, and, as mentioned above, similarity to previously marketed articles.

If further toxicological testing is necessary, the classification of medical devices provided in *Table 2* from general information chapter ⟨1031⟩ is important, because the degree and extent of toxicological testing that is required is strongly influenced by the nature and duration of the bodily contact with the article. The classification derived from *Table 2* in chapter ⟨1031⟩, coupled with the length of exposure to the article, is used in *Tables 3–5* of chapter ⟨1031⟩ to determine which toxicological tests need to be performed. *Table 2* below presents information extracted from *Tables 3–5* of chapter ⟨1031⟩ and indicates those circumstances for which sensitization testing should be considered.

Table 2. Articles For Which Sensitization Testing Should Be Considered Based on Article Category and Length of Exposure

Device Category	Body Contact	Contact Duration
Surface devices	Skin	A[a], B[b], C[c]
	Mucosal membrane	A, B, C
	Breached or compromised surfaces	A, B, C
External communicating devices	Blood path, indirect	A, B, C
	Tissue, bone, or dentin communicating	A, B, C
	Circulating blood	A, B, C
Implant devices	Tissue or bone	A, B, C
	Blood	A, B, C

[a] A: limited (less than 24 hours)
[b] B: prolonged (24 hours to 30 days)
[c] C: permanent (more than 30 days)

There are nine test methodologies reviewed in this chapter. *Table 3* lists the methods and the species with which they are performed.

Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test

Test	Species Used in Test
Magnusson & Kligman Maximization	Guinea pig

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Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test *(continued)*

Test	Species Used in Test
Standard Buehler	Guinea pig
Open Epicutaneous	Guinea pig
Freund's Complete Adjuvant	Guinea pig
Optimization	Guinea pig
Split Adjuvant	Guinea pig
Local Lymph Node Assay	Mouse
Mouse Ear Swelling	Mouse
Vitamin A Enhancement	Mouse

Given the preponderance of testing performed with either the *Magnusson & Kligman Guinea Pig Maximization Test* (GPMT) or *Buehler Tests* (BT), those tests will be reviewed in detail in this chapter. A brief summary of the remaining tests is provided as alternatives to the more frequently used procedures.

Each test should be periodically validated in the performing laboratory using positive controls such as hexyl cinnamic aldehyde, mercaptobenzothiazole, or benzocaine (positive controls recommended by the Organization for Economic Cooperation and Development [OECD]).

MAGNUSSON & KLIGMAN GUINEA PIG MAXIMIZATION TEST (GPMT)

Animals

Either male and female albino guinea pigs or both may be used. All animals should be in good health and weigh between 300 g and 500 g at the start of the experiment. The females should not be pregnant, nor should they have borne young previously. Prior to use, it is essential to acclimatize the animals to the laboratory conditions for at least 5 days. All animals should be handled in accordance with the guidelines in the appropriate regulatory requirements established for the humane treatment of animals. At least 10 test animals and 5 control animals should be used. To obtain sufficient analytical power (i.e., to detect weak sensitizers) it may be necessary to use 20 test animals and 10 control animals. Additional animals may be required to establish the proper doses to administer (see *Determination of Test Article Concentration*).

Housing and Feeding

The animal room should be held at 20 ± 3°, at 30% to 70% relative humidity, with 12 hours of light and dark. Animals may be housed individually or in group housing. Standard laboratory diets may be used (those satisfactory for guinea pigs ensure an adequate amount of ascorbic acid). Drinking water should be available ad libitum.

Animal Pretest Preparation

Animals should be randomized via a validated randomization method. For example, such methods may utilize random number tables or computer-generated random numbers. Sites on the animals intended for test article application (intrascapular region) should have the hair removed in a manner that does not abrade the skin. This may be accomplished via clipping, shaving, or with chemical depilatories. The chemical depilatory must not elicit irritation of its own. General observations of the animals prior to use in the test should be recorded, including any indication of ill health (do not use such animals in tests), and body weights.

Test Article Preparation[1]

The use of this test requires that the test article can be injected intradermally. When the test article is not suitable for direct administration, extracts should be prepared according to the procedure provided in general chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩.

Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of a GPMT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be injected intradermally (0.1 mL per site), using the solvent that will be employed in the *Test Procedure.* The concentration that causes only mild to moderate irritation (no

[1] For further information on sample preparation, see ANSI/AAMI/ISO/CEN Standard 10993–12—1996: Biological Evaluation of Medical Devices—Part 12: Sample Preparation and Reference Materials.

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extensive skin destruction, with no evidence of overt systemic toxicity to the animals) should be used in the *Intradermal Injection Induction Phase* of the *Test Procedure*.

Using two or more animals, apply via occlusive dressings and patches, a range of concentrations of test article or extracts of the article. Remove the dressings/patches after 24 hours, and examine the sites for erythema. Choose the concentration that causes only slight erythema for the *Topical Application Induction Phase* of the *Test Procedure*. Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure*. If the irritation threshold is not reached, then select the highest possible concentration for the *Topical Application Induction Phase* and *Challenge Phase* of the *Test Procedure*.

Test Procedure

INTRADERMAL INJECTION INDUCTION PHASE

This phase requires three pairs of injections administered intradermally, with the test and control injection of each pair on opposite sides intrascapularly. Each injection should contain 0.1 mL, with injection pairs 1 and 2 administered nearer to the head, and injection pair 3 administered slightly farther towards the tail. The pairs are nominally within an area of 8 cm^2. The pairs of injections consist of the following:

Injection pair 1:	A 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA), an oil–water emulsion containing mycobacteria, and the appropriate solvent/vehicle (see *Biological Reactivity Tests, In Vivo* ⟨88⟩). Control animals receive a mixture of FCA and physiological saline (1:1).
Injection pair 2:	The *Test Article Preparation* in the concentration as specified in *Determination of Test Article Concentration,* using the appropriate solvent/vehicle. Control animals receive only the solvent/vehicle.
Injection pair 3:	The *Test Article Preparation* in the concentration as specified in *Determination of Test Article Concentration* in a 1:1 (v/v) mixture with FCA. Control animals receive an injection of a 1:1 (v/v) mixture of FCA and solvent/vehicle.

TOPICAL APPLICATION INDUCTION PHASE

Seven days (±1 day) after completion of the *Intradermal Injection Induction Phase,* administer the test sample by topical application to the intrascapular region of each animal. For both test and control animals, if the *Test Article Preparation* does not cause skin irritation, apply 10% sodium lauryl sulfate in petrolatum approximately 24 hours before the start of the *Topical Application Induction Phase* to induce a local irritation.

Test animals should have 2- × 4-cm pieces of filter paper or absorbent gauze fully loaded with the *Test Article Preparation* (prepared within 24 hours of use) using the concentration selected in *Determination of Test Article Concentration* applied to each injection site. The filter paper or absorbent gauze should be secured to the animals using occlusive dressings. Control animals receive the same treatment, except that the appropriate solvent/vehicle is used instead of the test article.

Remove the dressings and patches approximately 48 hours after application.

CHALLENGE PHASE

This phase should occur 14 ± 1 days after the *Topical Application Induction Phase.* Hair should be removed from the test application sites. Filter paper patches or chambers are soaked with a freshly prepared *Test Article Preparation* in the concentration specified in *Determination of Test Article Concentration.* This is done for all test and control animals. The patches or chambers are secured with an occlusive dressing and removed after 24 ± 2 hours.

Observations

At approximately 24, 48, and 72 hours after removal of the challenge patches, the application sites should be examined for signs of reactions. Of particular importance are instances where the reaction of the test animals exceeds that of the control animals. All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. A true edematous reaction will blanch under gentle pressure. The longer the period of blanching, the greater the severity of edema.

Interpretation

There is more than one way of evaluating and grading the results from GPMT. *Tables 4*, *5*, and *6* list details for three such grading systems. Grades of 1 or higher in the test animals, with grades of less than 1 in control animals, are indicative of sensitization. If control animals display grade 1 reactivity, and if the test animals display reactivity above the greatest reactivity seen in the control animals, sensitization due to the test article is again suspected. The percentages in *Table 4* need to be revised if there are only 10 test animals (i.e., the categories would be 0, <10%, 10%–30%, 31%–60%, 61%–80%, and 81%–100%.) If there are 20 test animals, then multiples of 5% are appropriate.

Table 4. Classification Based on Percent of Responsive Test Animals

% of Positives in Test Group	Assigned Grade Class	
0	—	Nonsensitizer
<8	1	Weak

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Table 4. Classification Based on Percent of Responsive Test Animals *(continued)*

% of Positives in Test Group	Assigned Grade Class	
8–28	2	Mild
29–64	3	Moderate
65–80	4	Strong
81–100	5	Extreme

Table 5. Classification Based on Erythema and Edema Formation

Erythema and Eschar	Grade
No erythema	0
Slight or equivocal erythema	<1
Well-defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Edema	
No edema	0
Slight or equivocal edema	<1
Well-defined edema	2
Moderate edema	3
Severe edema	4

Table 6. Classification Based on Erythema Formation Alone

Erythema formation	Grade
No erythema	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

The results should be submitted for statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

Rechallenge

The extent of any response in the negative control group, under experimental conditions, shows the irritation potential of the *Test Article Preparation*. In this case, test and control animals should be rechallenged 1 week later on the untreated side of the animal, with a reduced concentration of the *Test Article Preparation*. A sensitized guinea pig will react to some degree to both challenges. A weak reaction occurring at a single time point in only one challenge should cast strong doubt as to whether that guinea pig is truly sensitized.[2]

STANDARD BUEHLER TESTS (SBT)

Animals

See *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

Housing and Feeding

See *Housing and Feeding* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

[2] Basketter D.A. Guinea pig predictive tests for contact hypersensitivity. In Immunotoxicology and Immunopharmacology, 2nd ed.; Dean, J.H, Luster, M.I., Munson, A.E., Kimber, I., Eds; Raven Press, Ltd: New York, 1994; pp 693–702.

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Animal Pretest Preparation

See *Animal Pretest Preparation* under *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*. The fur of the guinea pig may be removed from one flank by clipping.

Test Article Preparation

See *Test Article Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of an SBT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be applied using patches (for example, four 4 cm^2 absorbent pads) or chambers. The patches should be held in place using tape (if necessary) and occlusive dressings. The patches should be removed after approximately 6 hours, and any residues of the test chemical are removed from the test site. Observations are made at that time, and at 24 and 48 hours.

The concentration that causes only mild to moderate irritation (slight erythema, with no evidence of overt toxicity to the animals) and can be applied repeatedly to the same site should be used in the *Induction Phase* of the *Test Procedure*. Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure*.

Test Procedure

INDUCTION PHASE

Apply 0.4 mL of the *Test Article Preparation* in an appropriate solvent/vehicle at the dose identified in *Determination of Test Article Concentration*. Use patches similar to those used in *Determination of Test Article Concentration*. The patches should be applied to one flank (hair clipped off) and held in place occlusively for 6 hours. The animals may need to be restrained to ensure occlusion. Patches and any visible residues should be removed after 6 hours. Control animals also receive patches, but these contain only the appropriate solvent/vehicle. This process should be repeated three times a week for both test and control animals on the same site for three consecutive weeks (weekly intervals are used in the modified Buehler Test).

CHALLENGE PHASE

This phase should be carried out 14 days after the last application of the *Induction Phase*. Clip the hair off the previously untested flank of each animal 24 hours before the challenge application. As in the *Induction Phase,* apply patches containing the test article (concentration specified in *Determination of Test Article Concentration*) or solvent/vehicle alone to the untested areas of the test and control animals. To obtain well-defined edges at the application sites, commercial chambers with a lipped edge are preferred. Secure the patches with occlusive dressings, and keep them in place for 6 hours. Remove all patches after 6 hours.

OBSERVATIONS

At 22 ± 2 hours after removal of the patches, the application sites should have the animal's fur removed via clipping or depilation. After approximately 2 more hours, grade the sites (*Tables 4*, *5* or *6* may be employed). All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. Repeat the grading once again after 24 to 48 hours more have elapsed. The response of the test group versus the control group can be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

INTERPRETATION

The results should be submitted for a statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant.

See *Interpretation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

RECHALLENGE

See Rechallenge in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

OTHER SENSITIZATION TEST PROCEDURES

The *Magnusson & Kligman Guinea Pig Maximization Test* and the *Standard Buehler Tests* are the most frequently performed sensitization tests. However, there are a number of other methods that may be useful in the assessment of the potential for sensitization. Some may be applicable to both solid test articles and extracts, some only to extracts.

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Where the use of guinea pigs is called for in the following tests, the animals and their housing should meet the requirements as specified for *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test*. The fur of the guinea pig should be removed from test sites as indicated for *Animal Pretest Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test*.

Draize Test

This was the first predictive test accepted by the regulatory agencies, and is still in use. The test uses guinea pigs and the test article is administered via intradermal injections.

TEST ARTICLE PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material would need to be made. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the preparation procedure.

INDUCTION PHASE

One flank of each of 20 guinea pigs is shaved, then 0.05 mL of a 0.1% solution of test article is injected into the anterior flank. The next day, and then every other day thereafter up to day 20, 0.1 mL of the test article is injected into a new site on the same flank.

CHALLENGE PHASE

This phase begins 2 weeks after the final injection of the *Induction Phase*. The untreated flank is shaved, then 0.05 mL of test article is injected into each of the 20 guinea pigs. Twenty previously untreated animals serve as the controls, and receive injections of the test article as well.

OBSERVATIONS

The test sites of all control and test animals are evaluated for erythema at 24 and 48 hours after the challenge injections. The degree of reaction in test animals is compared to the reaction in control animals. A larger and/or more intense response by the test animals versus the control animals is indicative of sensitization.

Open Epicutaneous Test

This test uses guinea pigs. The goal is to determine the dose required to induce sensitization by simulating human usage via topical application of the test article.

TEST MATERIAL PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material need to be made. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the preparation procedure.

PRELIMINARY TESTING

A series of concentrations of test article is applied to 2 cm^2 areas of skin on the anterior flank of 6 to 8 guinea pigs (0.025 mL per application). The test sites should be examined for erythema 24 hours after test article administration. The highest concentration that does not cause irritation (maximum nonirritant concentration) and the lowest concentration causing erythema in approximately 25% of the animals (minimum irritant concentration) are determined.

INDUCTION PHASE

The test article (or control vehicle) is applied to 8 cm^2 areas of the flank skin of 6 to 8 guinea pigs daily for 3 weeks, or five times a week for 4 weeks. The amount per application is 0.01 mL. A set of increasing concentrations is again employed, ranging from the minimum irritant concentration using a stepwise progression. The test article should be applied to the same sites each time, unless irritation develops, in which case a new site on the same flank should be used. Control animals receive the same series of treatments using the vehicle instead of the test article.

CHALLENGE PHASE

Each animal is challenged on the untreated flank 24 to 72 hours after the last *Induction Phase* treatment using 0.025 mL applied to 2 cm^2 areas. A set of increasing concentrations is used, from minimum irritant concentration to the maximum nonirritant concentration, and five lower concentrations are also used.

OBSERVATIONS

The test sites are evaluated at 24, 48, and 72 hours post-treatment. The maximum concentration that does not cause irritation in the control group is determined. Animals from the test groups that develop inflammatory responses at concentrations lower than the maximum nonirritating concentration in the controls should be considered to be sensitized.

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Freund's Complete Adjuvant Test

This test is based upon the use of intradermal injections using the test article in a mixture of Freund's complete adjuvant and distilled water (50:50).

TEST MATERIAL PREPARATION

Because this test uses intradermal injections, extracts of the test material need to be made in order to use this procedure. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

PRELIMINARY TESTING

The minimum irritating and the maximum nonirritating concentrations are determined in the same manner as for *Preliminary Testing* in the *Open Epicutaneous Test.*

INDUCTION PHASE

The test area consists of six 2 cm^2 areas across the shoulders of the guinea pigs. Two groups of 10 to 20 guinea pigs each should be used. The test group animals are injected intradermally with 0.1 mL of a 5% solution of the test article extract in FCA/water. Control animals receive injections with FCA/water without the test article. These injections are repeated every 4 days until a total of three injections have been given.

CHALLENGE PHASE

This phase should begin 2 weeks after the last injection of the *Induction Phase*. Topical applications of 0.025 mL of test article at the minimum irritating and the maximum nonirritating concentrations, plus two lower concentrations, are administered to 2 cm^2 areas of the shaved flank. The test sites should remain uncovered.

OBSERVATIONS

The test sites are examined for the presence of erythema 24, 48 and 72 hours after the topical applications. The minimum nonirritating concentration in the control animals should be determined. Those test animals that display erythema at concentrations lower than the minimum nonirritating concentration in the control animals should be considered to be sensitized.

Optimization Test

This test has some similarities to the older *Draize Test.* Unlike the *Draize Test,* however, this test uses both intradermal and topical treatments, and includes adjuvant for some induction injections.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

INDUCTION PHASE

Twenty test and 20 control guinea pigs are used. A total of 10 intradermal injections should be given to each animal. Test animals receive 0.1 mL of a mixture of 0.1% test article and 0.9% saline (50:50) on day 1, with one injection into a shaved flank, and another into a portion of shaved dorsal skin. Two and 4 days later, one intradermal injection of the test article in saline is given to eight new dorsal sites. Every other day during weeks 2 and 3, the test article is injected intradermally into 10 sites over the shoulders in a 50:50 mixture of saline and FCA. The same sequence of injections is given to the 20 control animals, except that no test article is included with the saline or saline/FCA injections.

CHALLENGE PHASE

Thirty-five days after the first injection, the animals are challenged topically with 0.1 mL of the 0.1% solution of test article in saline (for test animals). The control animals receive saline injections only. At 45 days after the first injection, a second topical challenge is given. A nonirritating concentration of test article (0.05 mL) is applied topically to a 1 cm^2 area of untreated skin. This site should then be covered with a 2 cm^2 piece of filter paper, after which an occlusive dressing should be applied. The patch should be removed after 24 hours.

OBSERVATIONS

Twenty-four hours after each injection during week 1, the thickness of a fold of skin over the injection sites for each animal should be measured using a caliper (mm), and the two largest cross-diameters of each erythematous reaction should be recorded (mm). The reaction volumes are calculated by multiplying the fold thickness by the products of the two cross-diameters (expressed as µL). The mean reaction (+1 SD) volume during week 1 should be calculated for each animal.

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Challenge reaction volumes are calculated for each animal following the injections at day 35. If an animal develops a challenge reaction volume greater than its mean reaction volume + 1 SD, it should be considered sensitized.

Following the patch testing challenge, the test sites are evaluated for erythema and edema. Evaluations should be made using *Table 5*.

The number of positive animals should be compared statistically with the pseudopositive control animals. This should be done for both intradermal injection results and patch testing results. The Fisher exact test may be used.

The results from the intradermal injections and the patch testing, following separate statistical analysis, may be combined and evaluated using *Table 7* in order to classify a test article as a strong, moderate, or weak sensitizer; or not a sensitizer.

Table 7. Classification Scheme for Test Articles Based on the Optimization Test

Intradermal % of Positive Animals	Patch Test % of Positive Animals	Classification
S*, > 75	and/or S, > 50	Strong sensitizer
S, 50–75	and/or S, 30–50	Moderate sensitizer
S, 30–50	N.S.*, 0–30	Weak sensitizer
N.S., 0–30	N.S., 0	Not a sensitizer

* S = significant; N.S. = not significant

Split Adjuvant Test

This test makes use of both FCA and skin damage. The test article is applied topically.

TEST MATERIAL PREPARATION

Because this test employs topical test article applications, the article can be either in solid or liquid form. If extracts are to be made, see chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩ for extraction procedures.

INDUCTION PHASE

Ten to 20 guinea pigs are used for both test and control groups. An area of back skin immediately behind the scapulas should be shaved to the extent that the skin becomes glistening. The shaved areas should then be treated with dry ice for 5 to 10 seconds. A dressing made of loose mesh gauze with stretch adhesive and a 2- × 2-cm opening should be placed over the treated area, then secured with adhesive tape. The test article (0.2 mL of viscous materials, 0.1 mL of liquids, or solid material) is placed within the opening in the dressing on top of the treated skin. Two layers of #2 filter paper should be placed over the test article, then backed by occlusive tape. Then the filter paper/occlusive backed material should be secured to the surrounding dressing with adhesive tape. After 2 days have passed, the filter paper should be lifted from the test sites, and the test article reapplied on the same site. The filter paper and backing should be secured once again. After 2 more days, the filter paper should be lifted and two injections of 0.075 mL of FCA should be administered into the edges of the test site. Then the test material is once again applied, and the filter paper/backing resecured. The test article should be reapplied once more on day 7 and the filter paper/backing resealed. On day 9, the filter paper and all associated dressing material should be removed.

CHALLENGE PHASE

On day 22 following the induction treatment, 0.5 mL of test material (or the solid article) should be applied to a 2-× 2-cm area of shaved midback. The test sites should be covered by filter paper and backed by adhesive tape. This should be held in place with an elastic bandage secured with adhesive tape. Control animals receive the same challenge phase treatment. The preparation should be removed after 24 hours.

OBSERVATIONS

Twenty-four, 48, and 72 hours after the removal of the challenge phase preparation, the test sites should be evaluated for erythema and edema. The grading scheme of *Table 5* could be employed.

Mouse Ear Swelling Test

There are a number of potential advantages in using mice versus guinea pigs for sensitization methods. The classic guinea pig tests tend to be costly and require a long time to complete. Moreover, with the dependence upon relatively subjective scoring based on edema and erythema, methodological robustness, and ruggedness may be questionable. This test uses mice and employs both topical exposures and injections.

ANIMALS

Female, 6- to 8-week old CF-1, Balb/c, or Swiss mice should be used. They may be group housed in direct bedding cages. Acclimatization should be for at least 5 to 7 days. Food (appropriate mouse feed) and water should be available ad libitum. No

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animals with damaged pinnae should be used in the study. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

PRELIMINARY TESTING

The minimally irritating and maximally nonirritating concentrations of test article for this procedure should be determined. This is done by using four groups of two mice and examining the effects of at least four concentrations of test article.

INDUCTION PHASE

The abdomens of the animals should be shaved, then tape-stripped using a surgical adhesive tape until the test area is glistening. A single injection of 0.05 mL of FCA is subdivided into two injection sites administered intradermally within the shaved/stripped area, but along the borders. After the adjuvant injections, 100 µL of test article (using the minimally irritating concentration) or vehicle (controls) is applied to the center of the shaved test areas. After the test areas dry, the mice should be returned to their cages. The tape stripping and application of test article (but not FCA) is repeated each day for the next 3 days.

CHALLENGE PHASE

This phase should occur 7 days after the final topical induction application. The test article (highest nonirritating concentration) should be applied topically (20 µL) to one ear, while the opposite ear receives 10 µL of vehicle alone. This should be done for both test and control animals.

OBSERVATIONS

The thickness of both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). A sensitized animal is one in which the test article-treated ear is at least 20% thicker than its opposite ear. For the test to be valid, the test article-treated ears of control animals should not be more than 10% thicker than the opposite ears. If the control animal ears do not meet the requirements, the test should be repeated using lower concentrations.

Local Lymph Node Test

This test is based on the observation that exposure of the mice to sensitizers can cause hyperplasia of T cells within the auricular lymph nodes of mice. The method combines both in vivo and in vitro phases, and requires the use of radioisotopes. An unusual aspect of this test is that no challenge phase is required.

ANIMALS

Four groups of four mice at least, male or female CBA/ca mice (only one sex in a given test) between the ages of 8 to 12 weeks should be used.

TEST MATERIAL PREPARATION

Although in theory one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

PRELIMINARY TESTING

A nontoxic concentration of test article should be used. If not already established, a preliminary test for overt toxicity may be required to establish a suitable dose.

INDUCTION PHASE

Twenty-five µL of the appropriate test article concentration, or vehicle (controls), should be applied to the dorsal surface of each pinna for 3 consecutive days. Five days after the first treatment, the animals should be injected, via the tail vein, with 2.5 mL of phosphate buffered saline containing 20 µCi of ^{3}H-methyl thymidine. Five hours after the isotopic injection, the animals should be euthanized. The draining auricular lymph nodes should be removed from each animal of each test and control group. The nodes from all animals within a given group should be combined, such that a single cell suspension can be made from each group of animals. The cell suspension can be made by passing the nodes through a 200-mesh stainless steel gauze using a syringe plunger. The cells should then be centrifuged at 190 × g for 10 minutes, resuspended in 3 mL of 5% trichloroacetic acid (TCA), and held overnight at 4°.

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The resulting precipitate should be recovered by centrifugation, and the pelleted precipitate should be resuspended in 1 mL of 5% TCA. The suspension should then be placed in scintillation vials with 10 mL of scintillation fluid, and the disintegrations/minute (dpm) counted with a β-counter.

OBSERVATIONS

The ratio of dpm for each test group should be compared to the dpm for the control group. If the ratio equals or exceeds 3 for any test group, the concentration of test article used with that group may be considered to be sensitizing.

Vitamin A Enhancement Test

This test is similar to the *Mouse Ear Swelling Test* in that test articles are applied topically to the abdomen, with a challenge application to the ears, followed by measurements of ear thickness. A principal difference is the use of mouse feed supplemented with vitamin A acetate. The purpose of the supplementation is to increase the reactivity of the immune system, thereby increasing the potential sensitization reaction.

ANIMALS

Male, 3- to 4-week old Balb/c mice should be maintained on a diet supplemented with vitamin A acetate. The diet may be prepared by mixing each kg of feed with 0.477 g of gelatinized vitamin A acetate. The feed mixture should be used within 3 weeks of preparation. Mice intended for use in sensitization studies should have been on the supplemented diet for at least 4 weeks. The mice at the time of the sensitization study should therefore be between 7 and 10 weeks old. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

Although, in theory, one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction.

PRELIMINARY TESTING

The maximally nonirritating dose and minimally irritating concentrations should be determined using separate groups of animals. This could be done as described for *Preliminary Testing* in the *Mouse Ear Swelling Test.*

INDUCTION PHASE

The fur of the abdomen and thorax of 10 mice per group should be shaved. Then 100 µL of test article (at the minimally irritating concentration) should be applied to the test areas on days 0, 2, 4, 7, and 11. Control animals receive 100 µL of vehicle alone on the same schedule.

CHALLENGE PHASE

This phase should occur 4 days after the final application of the *Induction Phase.* Twenty-five µL of test article (at the maximally nonirritating concentration) should be applied to each ear of each animal in the test and control groups.

OBSERVATIONS

Ear thickness for both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). The percent increase in ear thickness should be calculated for each ear by subtracting the pretreatment measurement from the post-treatment measurement, dividing the result by the pretreatment measurement, then multiplying by 100. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test could be used for the comparison.)

The results of individual animals should also be calculated. If an increase in ear thickness for an animal from the test group is at least 50% greater than the largest increase of a control animal, that is indicative of sensitization. As an overall evaluation, should the results of the study provide a significant result of the statistical test at $p < 0.01$ for the control versus test group comparisons, or if at least two test animals have ear thickness increases in excess of 50% of the maximum control thickness changes and the group comparison showed a $p < 0.05$, sensitization is indicated for the test article.

⟨1191⟩ STABILITY CONSIDERATIONS IN DISPENSING PRACTICE

[NOTE—Inasmuch as this chapter is for purposes of general information only, no statement in the chapter is intended to modify or supplant any of the specific requirements pertinent to Pharmacopeial articles, which are given elsewhere in this Pharmacopeia.]

Aspects of drug product stability that are of primary concern to the pharmacist in the dispensing of medications are discussed herein.

Pharmacists should avoid ingredients and conditions that could result in excessive physical deterioration or chemical decomposition of drug preparations, especially when compounding (see *Pharmaceutical Compounding—Nonsterile Preparations*

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⟨795⟩). The stability and clinical effect of manufactured dosage forms can be greatly compromised by seemingly negligible alterations or inappropriate prescription compounding. Pharmacists should establish and maintain compounding conditions that include the ensuring of drug stability to help prevent therapeutic failure and adverse responses.

Stability—Stability is defined as the extent to which a product retains, within specified limits, and throughout its period of storage and use (i.e., its shelf-life), the same properties and characteristics that it possessed at the time of its manufacture. Five types of stability generally recognized are shown in the accompanying table.

Criteria for Acceptable Levels of Stability

Type of Stability	Conditions Maintained Throughout the Shelf Life of the Drug Product
Chemical	Each active ingredient retains its chemical integrity and labeled potency, within the specified limits.
Physical	The original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability, are retained.
Microbiological	Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits.
Therapeutic	The therapeutic effect remains unchanged.
Toxicological	No significant increase in toxicity occurs.

FACTORS AFFECTING PRODUCT STABILITY

Each ingredient, whether therapeutically active or pharmaceutically necessary, can affect the stability of drug substances and dosage forms. The primary environmental factors that can reduce stability include exposure to adverse temperatures, light, humidity, oxygen, and carbon dioxide. The major dosage form factors that influence drug stability include particle size (especially in emulsions and suspensions), pH, solvent system composition (i.e., percentage of "free" water and overall polarity), compatibility of anions and cations, solution ionic strength, primary container, specific chemical additives, and molecular binding and diffusion of drugs and excipients. In dosage forms, the following reactions usually cause loss of active drug content, and they usually do not provide obvious visual or olfactory evidence of their occurrence.

Hydrolysis

Esters and β-lactams are the chemical bonds that are most likely to hydrolyze in the presence of water. For example, the acetyl ester in aspirin is hydrolyzed to acetic acid and salicylic acid in the presence of moisture, but in a dry environment the hydrolysis of aspirin is negligible. The aspirin hydrolysis rate increases in direct proportion to the water vapor pressure in an environment.

The amide bond also hydrolyzes, though generally at a slower rate than comparable esters. For example, procaine (an ester) will hydrolyze upon autoclaving, but procainamide will not. The amide or peptide bond in peptides and proteins varies in the lability to hydrolysis.

The lactam and azomethine (or imine) bonds in benzodiazepines are also labile to hydrolysis. The major chemical accelerators or catalysts of hydrolysis are adverse pH and specific chemicals (e.g., dextrose and copper in the case of ampicillin hydrolysis).

Epimerization

Members of the tetracycline family are most likely to incur epimerization. This reaction occurs rapidly when the dissolved drug is exposed to a pH of an intermediate range (higher than 3), and it results in the steric rearrangement of the dimethylamino group. The epimer of tetracycline, epitetracycline, has little or no antibacterial activity.

Decarboxylation

Some dissolved carboxylic acids, such as *p*-aminosalicylic acid, lose carbon dioxide from the carboxyl group when heated. The resulting product has reduced pharmacological potency.

β-Keto decarboxylation can occur in some solid antibiotics that have a carbonyl group on the β-carbon of a carboxylic acid or a carboxylate anion. Such decarboxylations will occur in the following antibiotics: carbenicillin sodium, carbenicillin free acid, ticarcillin sodium, and ticarcillin free acid.

Dehydration

Acid-catalyzed dehydration of tetracycline forms epianhydrotetracycline, a product that both lacks antibacterial activity and causes toxicity.

Oxidation

The molecular structures most likely to oxidize are those with a hydroxyl group directly bonded to an aromatic ring (e.g., phenol derivatives such as catecholamines and morphine), conjugated dienes (e.g., vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (e.g., flavorings). Products of oxidation usually lack

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therapeutic activity. Visual identification of oxidation, for example, the change from colorless epinephrine to its amber colored products, may not be visible in some dilutions or to some eyes.

Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metal ions (e.g., copper and iron), and exposure to oxygen and UV illumination. The latter two causes of oxidation justify the use of antioxidant chemicals, nitrogen atmospheres during ampul and vial filling, opaque external packaging, and transparent amber glass or plastic containers.

Photochemical Decomposition

Exposure to, primarily, UV illumination may cause oxidation (photo-oxidation) and scission (photolysis) of covalent bonds. Nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions.

Ionic Strength

The effect of the total concentration of dissolved electrolytes on the rate of hydrolysis reactions results from the influence of ionic strength on interionic attraction. In general, the hydrolysis rate constant is inversely proportional to the ionic strength with oppositely charged ions (e.g., drug cation and excipient anions) and directly proportional to the ionic strength with ions of like charge. A reaction that produces an ion of opposite charge to the original drug ion because of the increasing ionic strength, can increase the drug hydrolysis rate as the reaction proceeds. High ionic strength of inorganic salts can also reduce the solubility of some other drugs.

pH Effect

The degradation of many drugs in solution accelerates or decelerates exponentially as the pH is decreased or increased over a specific range of pH values. Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions. A drug solution or suspension, for example, may be stable for days, weeks, or even years in its original formulation, but when mixed with another liquid that changes the pH, it degrades in minutes or days. It is possible that a pH change of only 1 unit (e.g., from 4 to 3 or 8 to 9) could decrease drug stability by a factor of 10 or greater.

A pH buffer system, which is usually a weak acid or base and its salt, is a common excipient used in liquid preparations to maintain the pH in a range that minimizes the drug degradation rate. The pH of drug solutions may also be either buffered or adjusted to achieve drug solubility. For example, pH in relation to pKa controls the fractions of the usually more soluble ionized and less soluble nonionized species of weak organic electrolytes.

The influence of pH on the physical stability of two phase systems, especially emulsions, is also important. For example, intravenous fat emulsion is destabilized by acidic pH.

Interionic (Ion^{N+}–Ion^{N-}) Compatibility

The compatibility or solubility of oppositely charged ions depends mainly on the number of charges per ion and the molecular size of the ions. In general, polyvalent ions of opposite charge are more likely to be incompatible. Thus, an incompatibility is likely to occur upon the addition of a large ion with a charge opposite to that of the drug.

Solid State Stability

Solid state reactions are relatively slow; thus, stability of drugs in the solid state is rarely a dispensing concern. The degradation rate of dry solids is usually characterized by first-order kinetics or a sigmoid curve. Therefore, solid drugs with lower melting point temperatures should not be combined with other chemicals that would form a eutectic mixture.

When moisture is present, the solid drug decomposition may change to zero-order chemical kinetics because the rate is controlled by the relatively small fraction of the drug that exists in a saturated solution, which is located (usually imperceptibly) at the surface or in the bulk of the solid drug product.

Temperature

In general, the rate of a chemical reaction increases exponentially for each 10° increase in temperature. This relationship has been observed for nearly all drug hydrolysis and some drug oxidation reactions. The actual factor of rate increase depends on the activation energy of the particular reaction. The activation energy is a function of the specific reactive bond and the drug formulation (e.g., solvent, pH, additives). As an example, consider a hydrolyzable drug that is exposed to a 20° increase in temperature, such as that from cold to controlled room temperature. The shelf life of the drug at controlled room temperature should be expected to decrease to one-fourth to one-twenty-fifth of its shelf life under refrigeration.

The pharmacist should also be aware that inappropriately cold temperatures may cause harm. For example, refrigeration may cause extreme viscosity in some liquid drugs and cause supersaturation in others. Freezing may either break or cause a large increase in the droplet size of emulsions; it can denature proteins; and in rare cases, it can cause less soluble polymorphic states of some drugs to form.

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STABILITY STUDIES IN MANUFACTURING

The scope and design of a stability study vary according to the product and the manufacturer concerned. Ordinarily the formulator of a product first determines the effects of temperature, light, air, pH, moisture, trace metals, and commonly used excipients or solvents on the active ingredient(s). From this information, one or more formulations of each dosage form are prepared, packaged in suitable containers, and stored under a variety of environmental conditions, both exaggerated and normal. At appropriate time intervals, samples of the product are assayed for potency by use of a stability-indicating method, observed for physical changes, and, where applicable, tested for sterility and or for resistance to microbial growth and for toxicity and bioavailability. Such a study, in combination with clinical and toxicological results, enables the manufacturer to select the optimum formulation and container and to assign recommended storage conditions and an expiration date for each dosage form in its package.

Responsibility of Pharmacists

Pharmacists help to ensure that the products under their supervision meet acceptable criteria of stability by (1) dispensing oldest stock first and observing expiration dates, (2) storing products under the environmental conditions stated in the individual monographs, labeling, or both, (3) observing products for evidence of instability, (4) properly treating and labeling products that are repackaged, diluted, or mixed with other products, (5) dispensing in the proper container with the proper closure, and (6) informing and educating patients concerning the proper storage and use of the products, including the disposition of outdated or excessively aged prescriptions.

ROTATION OF STOCK AND OBSERVANCE OF EXPIRATION DATES

Proper rotation of stock is necessary to ensure the dispensing of suitable products. A product that is dispensed infrequently should be closely monitored so that old stocks are given special attention, particularly with regard to expiration dates. The manufacturer can guarantee the quality of a product up to the time designated as its expiration date only if the product has been stored in the original container under recommended storage conditions.

STORAGE UNDER RECOMMENDED ENVIRONMENTAL CONDITIONS

In most instances, the recommended storage conditions are stated on the label, in which case it is imperative to adhere to those conditions. They may include a specified temperature range or a designated storage place or condition (e.g., "refrigerator," or "controlled room temperature") as defined in the *Packaging and Storage Requirements* ⟨659⟩. Supplemental instructions, such as a direction to protect the product from light, also should be followed carefully. Where a product is required to be protected from light and is in a clear or translucent container enclosed in an opaque outer covering, such outer covering is not to be removed and discarded until the contents have been used. In the absence of specific instructions, the product should be stored at controlled room temperature (see *Packaging and Storage Requirements* ⟨659⟩). The product should be stored away from locations where excessive or variable heat, cold, or light prevails, such as those near heating pipes or fluorescent lighting.

OBSERVING PRODUCTS FOR EVIDENCE OF INSTABILITY

Loss of potency usually results from a chemical change, the most common reactions being hydrolysis, oxidation-reduction, and photolysis. Chemical changes may also occur through interaction between ingredients within a product, or rarely between product and container. An apparent loss of potency in the active ingredient(s) may result from diffusion of the drug into, or its combination with, the surface of the container-closure system. An apparent gain in potency usually is caused by solvent evaporation or by leaching of materials from the container–closure system.

The chemical potency of the active ingredient(s) is required to remain within the limits specified in the monograph definition. Potency is determined by means of an assay procedure that differentiates between the intact molecule and its degradation products. Chemical stability data should be available from the manufacturer. Although chemical degradation ordinarily cannot be detected by the pharmacist, excessive chemical degradation sometimes is accompanied by observable physical changes. In addition, some physical changes not necessarily related to chemical potency, such as change in color and odor, formation of a precipitate, or clouding of solution, may serve to alert the pharmacist to the possibility of a stability problem. It should be assumed that a product that has undergone a physical change not explained in the labeling may also have undergone a chemical change, and such a product is never to be dispensed. Excessive microbial growth, contamination, or both, may also appear as a physical change. A gross change in a physical characteristic such as color or odor is a sign of instability in any product. Other common physical signs of deterioration of dosage forms include the following.

Solid dosage forms: Many solid dosage forms are designed for storage under low-moisture conditions. They require protection from environmental water and therefore should be stored in tight containers (see *Packaging and Storage Requirements* ⟨659⟩) or in the container supplied by the manufacturer. The appearance of fog or liquid droplets, or clumping of the product, inside the container signifies improper conditions. The presence of a desiccant inside the manufacturer's container indicates that special care should be taken in dispensing. Some degradation products, for example, salicylic acid from aspirin, may sublime and be deposited as crystals on the outside of the dosage form or on the walls of the container.

Hard and soft gelatin capsules: Since the capsule formulation is encased in a gelatin shell, a change in gross physical appearance or consistency, including hardening or softening of the shell, is the primary evidence of instability. Evidence of release of gas, such as a distended paper seal, is another sign of instability.

Uncoated tablets: Evidence of physical instability in uncoated tablets may be shown by excessive powder and/or pieces (i.e., crumbling as distinct from breakage) of tablet at the bottom of the container (from abraded, crushed, or broken tablets); cracks

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or chips in tablet surfaces; swelling; mottling; discoloration; fusion between tablets; or the appearance of crystals that obviously are not part of the tablet itself on the container walls or on the tablets.

Coated tablets: Evidence of physical instability in coated tablets is shown by cracks, mottling, or tackiness in the coating and the clumping of tablets.

Dry powders and granules: Dry powders and granules that are not intended for constitution into a liquid form in the original container may cake into hard masses or change color, which may render them unacceptable.

Powders and granules intended for constitution as suspensions: Dry powders and granules intended for constitution into solutions or suspensions require special attention. Usually such forms are antibiotics or vitamins that are particularly sensitive to moisture. Since they are always dispensed in the original container, they generally are not subject to contamination by moisture. However, an unusual caked appearance necessitates careful evaluation, and the presence of a fog or liquid droplets inside the container generally renders the preparation unfit for use. Presence of an objectionable odor also may be evidence of instability.

Effervescent tablets, granules, and powders: Effervescent products are particularly sensitive to moisture. Swelling of the mass or development of gas pressure is a specific sign of instability, indicating that some of the effervescent action has occurred prematurely.

Liquid dosage forms: Of primary concern with respect to liquid dosage forms are homogeneity and freedom from excessive microbial contamination and growth. Instability may be indicated by cloudiness or precipitation in a solution, breaking of an emulsion, nonresuspendable caking of a suspension, or organoleptic changes. Microbial growth may be accompanied by discoloration, turbidity, or gas formation.

Solutions, elixirs, and syrups: Precipitation and evidence of microbial or chemical gas formation are the two major signs of instability.

Emulsions: The breaking of an emulsion (i.e., separation of an oil phase that is not easily dispersed) is a characteristic sign of instability; this is not to be confused with creaming, an easily redispersible separation of the oil phase that is a common occurrence with stable emulsions.

Suspensions: A caked solid phase that cannot be resuspended by a reasonable amount of shaking is a primary indication of instability in a suspension. The presence of relatively large particles may mean that excessive crystal growth has occurred.

Tinctures and fluidextracts: Tinctures, fluidextracts, and similar preparations usually are dark because they are concentrated, and thus they should be scrutinized carefully for evidence of precipitation.

Sterile liquids: Maintenance of sterility is of course critical for sterile liquids. The presence of microbial contamination in sterile liquids usually cannot be detected visually, but any haze, color change, cloudiness, surface film, particulate or flocculent matter, or gas formation is sufficient reason to suspect possible contamination. Clarity of sterile solutions intended for ophthalmic or parenteral use is of utmost importance. Evidence that the integrity of the seal has been violated on such products should make them suspect.

Semisolids (creams, ointments, and suppositories): For creams, ointments, and suppositories, the primary indication of instability is often either discoloration or a noticeable change in consistency or odor.

Creams: Unlike ointments, creams usually are emulsions containing water and oil. Indications of instability in creams are emulsion breakage, crystal growth, shrinking due to evaporation of water, and gross microbial contamination.

Ointments: Common signs of instability in ointments are a change in consistency and excessive "bleeding" (i.e., separation of excessive amounts of liquid) and formation of granules or grittiness.

Suppositories: Excessive softening is the major indication of instability in suppositories, although some suppositories may dry out and harden or shrivel. Evidence of oil stains on packaging material should warn the pharmacist to examine individual suppositories more closely by removing any foil covering. As a general rule (although there are exceptions), suppositories should be stored in a refrigerator (see *Packaging and Storage Requirements* ⟨659⟩).

PROPER TREATMENT OF PRODUCTS SUBJECTED TO ADDITIONAL MANIPULATIONS

In repackaging, diluting a product or mixing it with another product, the pharmacist may become responsible for its stability.

Repackaging: In general, repackaging is inadvisable. However, if repackaging is necessary, the manufacturer should be consulted concerning potential problems. In the filling of prescriptions, it is essential that suitable containers be used. Appropriate storage conditions and, when appropriate, an expiration date and beyond use date should be indicated on the label of the prescription container. Single-unit packaging calls for care and judgment and for strict observance of the following guidelines: (1) use appropriate packaging materials, (2) if stability data on the new package are not available, repackage at any one time only sufficient stock for a limited time, (3) include on the unit-dose label a lot number and an appropriate beyond-use date, (4) if a sterile product is repackaged from a multiple-dose vial into unit-dose (disposable) syringes, discard the latter if not used within 24 hours, unless data are available to support longer storage, (5) if quantities are repackaged in advance of immediate need, maintain suitable repackaging records showing name of manufacturer, lot number, date, and designation of persons responsible for repackaging and for checking (see *General Notices*), (6) if safety closures are required, use container closure systems that ensure compliance with compendial and regulatory standards for storage.

Dilution or mixing: If a product is diluted, or if two products are mixed, the pharmacist should observe good professional and scientific procedures to guard against incompatibility and instability. For example, tinctures such as those of belladonna and digitalis contain high concentrations of alcohol to dissolve the active ingredient(s), and they may develop a precipitate if they are diluted or mixed with aqueous systems. Pertinent technical literature and labeling should be consulted routinely; it should be current literature, because at times formulas are changed by the manufacturer. If a particular combination is commonly used, consultation with the manufacturer(s) is advisable. Since the chemical stability of extemporaneously prepared mixtures is unknown, the use of such combinations should be discouraged; if such a mixture involves an incompatibility, the pharmacist might be responsible. Oral antibiotic preparations constituted from powder into liquid form should never be mixed with other products.

Combining parenteral products necessitates special care, particularly in the case of intravenous solutions, primarily because of the route of administration. This area of practice demands the utmost in care, aseptic technique, judgment, and diligence.

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Because of potential unobservable problems with respect to sterility and chemical stability, all extemporaneous parenteral preparations should be used within 24 hours unless data are available to support longer storage.

INFORMING AND EDUCATING THE PATIENT

As a final step in meeting responsibility for the stability of drugs dispensed, the pharmacist is obligated to inform the patient about the proper storage conditions (for example, in a cool, dry place—not in the bathroom) for both prescription and nonprescription products, and to suggest a reasonable estimate of the time after which the medication should be discarded. When beyond-use dates are applied, the pharmacist should emphasize to the patient that the dates are applicable only when proper storage conditions are observed. Patients should be encouraged to clean out their drug storage cabinets periodically.

⟨1195⟩ SIGNIFICANT CHANGE GUIDE FOR BULK PHARMACEUTICAL EXCIPIENTS

BACKGROUND

This general information chapter was derived from an international guidance on the evaluation of the significance of changes involving the manufacture of bulk pharmaceutical excipients. It is intended to assist excipient manufacturers in determining the need for informing the excipient user and regulatory authorities about the nature of the change.

The chapter provides minimum recommendations when considering the effect of a change in the manufacturing process on the excipient. When deciding how to use this chapter, each manufacturer must consider how it may apply to that manufacturer's product and processes. The diversity of excipients means that some principles of this chapter may not be applicable to certain products and processes.

This chapter is divided into several sections. The first section provides the general guidance necessary for evaluating a change and determining the necessity of informing the user and/or regulatory authorities. One section provides criteria for determining whether a change will involve a significant risk. Also included is a decision tree that is useful in considering the potential effect of a change on excipient performance.

INTRODUCTION

Purpose

This document is meant to establish uniform considerations for evaluating the significance of changes involving the manufacture of bulk pharmaceutical excipients (BPEs). The purpose of the evaluation is to determine the need for informing the excipient user and regulatory authorities about the nature of the change.

Scope

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients [referred to throughout this document as "excipient(s)"] intended for use in human drugs, veterinary drugs, and biologics. The principles set forth here must be applied once it has been determined that a chemical is intended for use as a component of a drug product. As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product should increase and should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩ should be applied and maintained. Judgment, based on risk analysis and a thorough knowledge of the process, is required to determine from which processing step the GMPs should be implemented.

Principles Adopted

This chapter should be of international application, bearing in mind that pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. It provides minimum recommendations when considering the impact of a change on the excipient. As an international guidance document, it cannot specify all national legal requirements nor cover in detail the particular characteristics of every excipient.

When considering how to use this chapter, each manufacturer should consider how it may apply to that manufacturer's product and processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes. The terminology "should" and "it is recommended" do not necessarily mean "must" as used in the application of this chapter.

Excipients may contain minor components that are known to be or might be necessary for the correct functioning of the excipient. The presence of these "essential concomitant components" in the excipient should not be construed as undesirable. These concomitant components are not considered part of the impurity profile, but should be considered separately. Water may be a concomitant component in some excipients, but may be included in the impurity profile for others. (See *Impurity Profile* in *Appendix 3* for more information.)

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Layout

This chapter is divided into several sections. The first section provides a background discussion necessary for evaluating a change and determining the necessity of informing the user and/or regulatory authorities. A second section provides criteria for determining the risk that a change will be significant, including guidance on development of an impurity profile. Also included are the following: The *Glossary* contains terms and definitions used in all sections of this document. *Appendix 1* includes some examples of changes that would be classified into each of the three risk levels. *Appendix 2* provides a decision tree useful in considering the potential impact of a change on excipient performance. *Appendix 3* delineates the development of an impurity profile.

GENERAL GUIDANCE

Differentiation of Excipient Manufacture

Evaluating the impact of a change in the manufacture of an excipient could be more difficult than that for an active pharmaceutical ingredient (API). Although the API is seldom used for more than a handful of therapeutic purposes, the BPE is often used with a broad range of active ingredients and in a diverse range of finished dosage forms. Whereas the API is typically of high purity and well characterized by the quality control and analytical laboratory, the BPE is often a natural substance, mixture, or polymer, the chemical and physical properties of which are more difficult to quantify. For a more thorough discussion of GMPs that apply to excipient manufacture, see the general information chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩.

Definition of Significant Change

Any change by the manufacturer of an excipient that alters an excipient's physical or chemical property outside the established limits, or that is likely to alter the excipient performance in the dosage form, is considered significant. Such changes may necessitate notifying the local regulatory authority if required. Regardless of whether there is a regulatory requirement, the manufacturer has an obligation to notify its users of a significant change so that the user can evaluate the impact of the change on the user's products. It is suggested that unless there is clear indication from evaluation of the change that it is not significant as stipulated by this general chapter, the pharmaceutical user should be notified.

The types of change that are considered here are changes to the following: site, scale, equipment, process, packaging and labeling, and specification (including raw materials).

The requirement for evaluating the impact of change on the excipient begins, at a minimum, with the raw materials for the first processing step from where GMP compliance begins. GMP requirements increase as the manufacturing process progresses. Thus, at some logical processing step, usually well before the final finishing operation, appropriate GMPs should be imposed and maintained throughout the remainder of the process. Methods such as HACCP (Hazard Analysis and Critical Control Point), FEMA (Failure Effects Mode Analysis), or a detailed process flow diagram may be used to identify the unit operations, required equipment, stages at which various substances are added, key steps in the process, critical parameters (time, temperature, pressure, etc.), and necessary monitoring points. Judgment, based on risk analysis and a thorough knowledge of the process, is required to determine at which processing step the GMP should be implemented.[1]

It is important to give careful consideration to any processing changes that occur after the excipient has been synthesized or isolated but prior to packaging. However, it must be recognized that a change made earlier in the process can result in a change in the excipient functionality, and it is recommended that such changes also be considered.

SIGNIFICANT CHANGE

Evaluation Criteria

These criteria, in the form of questions, are presented for consideration when evaluating the impact of a change relating to excipient manufacture:

1. Has there been a change in the chemical properties or composition of the excipient as a result of the change?
2. Has there been a change in the physical properties of the excipient as a result of the change?
3. Has there been a change in the "essential concomitant components" profile for the excipient as a result of the change?
4. Has there been a change in the functionality of the excipient as a result of the change?
5. Where applicable, has the moisture level changed?
6. Where applicable, has the bioburden changed?
7. Has there been a change in the origin of any raw materials or contact packaging?

An affirmative answer to any of these questions indicates that the impact of the change on the excipient may lead to changes in its performance in the dosage form.

It is important to provide objective criteria for evaluating when a change has occurred in an excipient property or composition, in the essential concomitant components profile, in biological origin, or in its functionality. This enables the BPE manufacturer to evaluate the significance of the change on the excipient for the purpose of notifying the regulatory authorities and/or the user.

[1] See chapter ⟨1078⟩, *GMPs For Excipients.*

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Determination of Significance

Criterion 1: Evaluation of the chemical properties or composition of an excipient should include, at a minimum, all monograph and manufacturer specification parameters. A comparison of these test results for the excipient before and after a change should be done to determine if there is a statistically significant difference.

Criterion 2: Physical properties should be considered based upon the physical form of the excipient and its functionality as known or as used by the end users. A physical property that is part of a mutually agreed-upon specification between the manufacturer and end user should also be evaluated. For example, a manufacturer of an excipient powder should consider measuring the impact of changes on such physical parameters as bulk density, surface area, particle shape, and particle size distribution. Liquid excipients might be evaluated for changes in their pH and viscosity. For all polymeric excipients, the effect of a change on a physical property, such as molecular weight distribution, should be considered.

Criterion 3: Objective criteria are also necessary when considering changes to the "essential concomitant components" profile for an excipient as a result of changes. The profile, as noted in *Appendix 3*, contains the following:

- identified organic impurities,
- unidentified organic impurities at or above 0.10%, whether specified or not,[2]
- residual solvents, and
- inorganic impurities

The feasibility of developing an impurity profile varies with the composition and origin of the excipient. It is important to note that identifying and quantifying impurities in some excipients are extremely difficult. Thus, an excipient manufacturer may not have developed an impurity profile. In that case, it is important for excipient manufacturers to either document their efforts to identify and quantify the impurities that may be present to justify their limited results or to justify other means by which changes may be evaluated.

The significance of the change can be determined by comparing the impurity profile of the pre-change material with that of the post-change product. Therefore, once the profile has been developed, it should be reassessed following changes to the process. An impurity should be monitored as part of the profile if it is present at or greater than 0.10%, if it has an established physiological effect, or if it is known to be unsafe at a lower level.

The content of the impurity profile varies with the nature of the excipient, the raw materials used in its manufacture, and its chemical composition. Where possible, changes are considered significant whenever a new impurity is introduced at the 0.10% concentration or higher, or when an impurity previously present at or greater than 0.10% disappears. Changes to the quantity of an existing impurity specified in a monograph and reported on the Certificate of Analysis (COA) should be treated as a chemical property for the purposes of this evaluation.

Changes in the levels of residual solvents should be considered when determining the significance of change. See *Residual Solvents* ⟨467⟩ for details.

Criterion 4: Objective criteria for evaluating changes to excipient functionality are desirable. However, the nature of this type of study can vary broadly based upon the excipient and its application in the dosage form. It must also be recognized that the excipient manufacturer does not always know each use of the excipient. Therefore this chapter cannot provide objective criteria for this study but stresses the importance of such a consideration by the manufacturer. If there is the potential that the functionality of the excipient may be affected by the change, users should be notified and material provided upon request so that they can determine the impact of the change in their finished pharmaceutical products.

Criterion 5: Often the excipient contains moisture, the presence of which can have an impact on excipient performance in the preparation of the pharmaceutical dosage form. Therefore a change in the moisture level beyond the range typical of production, even though within the compendial or specification limit, can affect its stability and/or end use.

Criterion 6: Change in the processing steps, raw materials, or equipment can adversely affect control of microorganisms in the excipient. Therefore the effect of the change on the bioburden should be evaluated, particularly for excipients susceptible to microbial growth.

Criterion 7: Change in the origin of a raw material or contact packaging can result in a change to the other six change criteria. Change in origin can involve the country of origin, geological origin, or species of origin for the raw material.

A change in the country of origin of a raw material or contact packaging material can affect the status of the excipient as it relates to the potential presence of bovine spongiform encephalopathies (BSEs), transmissible spongiform encephalopathy (TSE) material, or genetically modified organisms (GMOs). The country of origin of animal raw material, or of components used in the manufacture of the raw material, can result in noncompliance with relevant TSE regulations.[3,4] Current information on BSE and related diseases can be accessed on the United States Department of Agriculture (USDA) website (usda.gov).

A change in the geological origin of a mineral-based excipient can alter the composition of the excipient. Geological formations containing the same mineral can differ in their chemical composition, crystalline structure, density, etc. A change in geological origin of raw material can affect the excipient's chemical or physical properties, the impurity profile, or excipient functionality.

A change to the species of origin for raw materials of either animal or vegetable origin can raise concern. Switching from one animal species to another can affect the status of the excipient as it relates to the presence of BSE or TSE material in the excipient, as noted above. Switching from animal-derived to plant-derived raw materials, although eliminating the issue of BSE or TSE material, raises the potential for the presence of plant-based allergenic material in the excipient. Switching from one plant species to another also can result in the possible presence of an allergen in the excipient. In addition to this issue with

[2] It is recognized that while desirable, it may not be possible to achieve this for all excipients, particularly those of a more complex chemical nature, e.g. natural polymers, for which there may be no adequate means of determining related substances. However, the impurity profile documentation should demonstrate why this was not achievable.

[3] *European Pharmacopoeia*, General Text 5.2.8, *Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Medicinal Products.*

[4] U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Federal Register: November 4, 2003, Volume 68, Number 213 (Proposed Rules), 9 CFR Parts 93, 94, and 95, *Bovine Spongiform Encephalopathy; Minimal Risk Regions and Importation of Commodities.*

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allergens, use of plant-derived raw materials can affect pharmaceutical manufacturers who have a concern about the presence of GMOs in the excipient.

Risk Levels

In the evaluation of the effect of changes on the excipient, it is recognized that even with objective criteria, some judgment may be necessary. To facilitate the decision as to the significance of a change and the likely effect on the dosage form, the types of changes are classified using three levels:

- Level 1: Minor Change
- Level 2: Might Be Significant
- Level 3: Always Significant

LEVEL 1: MINOR CHANGE

These changes are considered unlikely to affect the excipient's chemical or physical properties, impurity profile, or functionality. Such changes should be documented, but notifications to the users and regulatory authorities are not necessary.

LEVEL 2: MIGHT BE SIGNIFICANT

The effect of the change should be evaluated against *Criterion 1, Criterion 2,* and *Criterion 3* (chemical and physical properties and impurity profile) to determine its potential effect on excipient functionality. A change in the biological origin of a raw material should be considered with regard to TSE or GMO regulations. A Level 2 change should always be communicated to the users and regulatory authorities.

LEVEL 3: ALWAYS SIGNIFICANT

This type of change should always be communicated to the users and regulatory authorities. Shipment of the changed excipient to the user should not occur without consent from the user's company.

Protocol Design

There should be a written protocol for the evaluation of a change to determine whether it is significant. The protocol should describe the nature of the change, the reason it may be significant, the testing to be performed to evaluate the change, and the criteria for determining the significance. If the change is attributable to a new biological source for raw materials used in the manufacture of the excipient, it is recommended that the regulatory status of the raw material (i.e., BSE/TSE, GMO agents) be evaluated first. Then, where possible, the results from the testing of a minimum of 10 pre- and 3 post-change batches of excipient should be compared (see *Supporting Data*, below). If significant changes are seen, then an assessment of the significance should be made.

The manufacturer should test the excipient made after the change for all specification properties and compare the results to the historical data. A standard statistical test, such as a *t*-test of the means, should be used to compare the new data with the historical data. If when using an appropriate statistical analysis there is sufficient evidence that the populations are different at the 95% confidence interval, the change should be considered significant. As an additional check on consistency, it is also recommended that the new batch specification properties be plotted on standard Statistical Quality Control (SQC) control charts, along with standard batch results.

Supporting Data

It is preferable to use data to measure the effect of a change on the excipient. The comparison should begin with chemical and physical properties, followed, where appropriate, by moisture, bioburden, impurity profile, and functionality. The manufacturer should use good judgment on sample comparisons for the other evaluations.

Chemical and physical properties lend themselves to quantitative measurement. Often these properties are part of the specification for the excipient. As such there should be a large body of test data to use for the properties affected for comparison to the corresponding data of the excipient made after the change.

Equivalence of impurity profiles is shown by comparing the data for the pre-change and post-change batches. If the following conditions are met, there has been no significant change in the impurity profile. [NOTE—*Residual Solvents* ⟨467⟩ notes that under certain circumstances an impurity concentration below 0.10% may be of concern and the excipient manufacturer should take this under consideration.]

1. No new impurity is present at or above 0.10%, nor has an impurity at this level disappeared that was previously in the impurity profile.
2. Residual solvent and impurities remain within the 95% confidence interval of the mean of the batches produced before the change.

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TYPES OF CHANGES

Site Change

A change in site can involve either the production or packaging of the excipient or its quality control testing. If the proposed manufacturing site was never used to produce the excipient, then the change poses a greater risk of altering the excipient's performance and is considered a *Level 3* change. If the proposed site was used for this purpose within the past year and the process, equipment, utilities, and raw materials are all unchanged, the risk is considered minor and thus a *Level 1* change. However, if the excipient was produced before at the proposed site with the same process, equipment, utilities, and raw materials more than 1 year ago, the risk is moderate and thus *Level 2*.

If the change involves the quality control laboratory, then the impact hinges on the test method. If the method remains the same, the change is a *Level 1*, provided a formal method transfer or validation is conducted. If the new lab uses a different analytical technique or analytical equipment, then the change should be evaluated more carefully, as required by a *Level 2* change.

Scale

Manufacturers often find ways to increase the scale of production. If the excipient is being scaled up from pilot to production, the change is likely to be significant and thus a *Level 3*. When the change in scale results from the use of new and larger, or smaller, production equipment using the same operating principle, which is often the case in batch processing, the change is a *Level 2*. If the existing equipment is optimized to increase capacity without altering the process, often found in continuous processing, the change is considered minor and treated as *Level 1* provided that a comparison of pre- and post-change data shows no statistically significant difference. However, careful consideration should be given to changes that are made that can clearly affect the properties of the excipient.

Equipment

The evaluation of equipment change concerns the issue of whether it is equivalent to the equipment it replaces. Generally, equipment that is a replacement in kind is considered a minor *Level 1* change. If the new equipment is not a replacement in kind but is included in the process validation, then the change is still a *Level 1*. Otherwise the change is considered *Level 3*.

Manufacturing Process

A change in process often involves changes to the processing instructions, such as target levels for such parameters as temperature, pressure, and flow rate; the raw materials to be used; the sequence of operating steps; and the operation to be performed, including reprocessing. As illustrated in the decision tree in *Appendix 2*, each type of process change can be further detailed.

If there is a change in a process parameter that is within the process validation, such as operating at a new target within the qualified range, then the change is a *Level 1*. However, if the process parameter is outside of the validation, then the change should be evaluated as a *Level 2*.

If minor changes are made to the processing steps, such as a small change outside of the validated range in the rate of addition of an ingredient, then the change is a *Level 2*. A major change, such as changing the point at which an ingredient is added to earlier or later in the process, is potentially significant and thus a *Level 3*.

Reprocessing of an excipient followed by a purification step, when not typical of the process, should be evaluated as a *Level 2* change. However, if no further purification of the bulk excipient occurs, this type of change is considered a *Level 3*.

Packaging and Labeling

These changes involve the package components meant for protection and distribution of the excipient. Any change in the package or packaging components such as the drum, box, liner, or tamper-evident seal that is a replacement in kind is a minor change *(Level 1)*. Replacement in kind applies to containers constructed of the same materials and sealed in a similar manner, and liners made of the same components. Any change that is not a replacement in kind should be evaluated as a *Level 3*. Any change to labeling pertaining to the site of manufacture or testing, the biological origin, additives, or storage and handling conditions should be evaluated as a *Level 3*.

Specifications

Differences in raw materials can be further defined by the supplier used, their specifications, biological origin, country of origin for those derived from animals, or the addition to or removal of the raw material from the BPE process. If the new supplier provides its raw material against a specification essentially the same as that of the former supplier and the raw material method of manufacture is similar, the change is minor and treated as *Level 1*. However, if the specifications, biological origin, or country of origin changes, or the manufacturing process is different, then the change should be evaluated as potentially significant *(Level 2)*. Also, any change of source for an animal-origin material should be treated as a *Level 2* change, if the source is determined to be not from a risk country as codified in 9 CFR 94.18. Finally, if the change in raw material involves the addition or removal of an ingredient from the process to produce or preserve the BPE or is otherwise used to produce the bulk excipient, the change is likely to be significant *(Level 3)*. Similar consideration should be given for any change in origin of raw materials that results in a potential that the raw material might contain risk materials (i.e., BSE, TSE, allergens, or GMOs).

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Changes are sometimes made to the excipient specification or the quality control test method. When changes are not the result of a monograph change, their significance should be evaluated. Such test or specification changes may be made to the excipient product or to the intermediate component. [NOTE—In some circumstances, relaxing the specification may lower the quality if the specification is for a significant property; therefore any change needs to be evaluated and its significance needs to be documented.]

Changes to an excipient specification or test method are *Level 3* changes. For example, adding a new specification parameter for the purpose of improving the quality of the excipient through lot selection is potentially a very significant *Level 3* change. If the specification change relaxes a specification parameter, the effect on excipient quality should be evaluated as a *Level 2* change. An example of a minor change is the additional testing of the excipient initiated with the sole purpose of further characterizing the material without altering its quality, and is a *Level 1* risk; however, notification is supported.

If a specification for a raw material from the same supplier(s) is made more stringent, then the change is unlikely to be significant *(Level 1),* whereas if the specification becomes less stringent, then the change should be evaluated carefully *(Level 2).*

When a change is made that either increases or maintains the level of process control in the manufacturing process, it should be treated as a *Level 1.* If the change in process control relaxes the control, then the effect should be carefully evaluated as *Level 2.* An illustrative example is pH control. If a new pH meter allows for more precise measurement, the process control is improved and the change falls under *Level 1.* However, if the pH control is relaxed by using a less precise measuring device, the change is treated as *Level 2.*

Multiple Changes

Multiple changes involve more than one change occurring simultaneously. The risk level for consideration of the impact of the changes should be the highest level for any single change. However, the effect of the totality of changes should also be assessed, because this may suggest that the overall risk is higher.

REPORTING REQUIREMENTS

Documentation

It is recommended that the evaluation of changes to the excipient be documented, regardless of the level of change. The report should indicate the basis for evaluating the effect of the change on the excipient, the significance of the data used in reaching the conclusion, and the actions taken.

Where appropriate, the process validation should be updated to reflect the changed process. This is clearly indicated where the evaluation has led to the conclusion that the change should be considered significant.

Notification

The user should be given as much advance notification of impending change as possible. For *Level 3* changes in particular, the user may require time to complete the evaluation of the impact of the change on their formulations. During this period the user may request inventory of the excipient produced before the change was made. The manufacturer should plan for the change with this eventuality in mind.

Regardless of the apparent level of the change, changes that are found to meet the definition of significant change resulting from the evaluation require user notification.

Regulatory authorities often require notification of significant changes involving the manufacture of excipients. Such notification should be done as required by the applicable authority.

GLOSSARY

Active Pharmaceutical Ingredient (API): Any substance or mixture of substances that is intended to be used in the manufacture of a drug (medicinal) product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

Batch Process: A manufacturing process that produces the excipient from a discrete supply of the raw materials that are present before the completion of the reaction.

Bioburden: The nature and quantity of microorganisms present in the excipient.

Biological Origin: Defined as either animal origin or nonanimal origin, based on the source of the raw material used in the manufacture of the excipient, and also includes materials that potentially come into contact with equipment used in the manufacture of other materials with animal-derived or GMO-derived components.

Bovine Spongiform Encephalopathy (BSE): A pathological brain deterioration condition of cattle believed to be caused by a prion that can be transmitted to cause variant Creutzfeldt-Jakob disease (vCJD) in humans.

Bulk Pharmaceutical Excipient (BPE): See *Excipient.*

Chemical Property: A quality parameter that is measured by chemical or physicochemical test methods.

Concomitant Component: A substance found in an excipient that is not the intended chemical entity, but may be necessary for ensuring the proper performance of the excipient in its intended use, and is not an impurity or a foreign substance (formerly referred to as a minor component).

Confidence Interval: A range, calculated from sample data, within which a population parameter, such as the population mean, is expected to lie, with a given level of confidence.

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Continuous Process: A manufacturing process that continually produces the excipient from a continuous supply of raw material.

Decision Tree: A visual presentation of the sequence of events that can occur, including decision points.

Drug Substance: See *Active Pharmaceutical Ingredient*.

Equipment: The implements used in the manufacture of an excipient.

Excipient: Any substance, other than the drug substance, in a drug product that has been appropriately evaluated for safety and is included in a drug delivery system to either aid the processing of the drug product during its manufacture; protect, support or enhance stability, bioavailability, or patient acceptability; assist in product identification; or enhance any other attribute of the overall safety and effectiveness of the drug product during storage or use.

Foreign Substance: A component that is present in the BPE but that is *not* introduced into the excipient as a consequence of its synthesis or purification and is not necessary to achieve the required functionality (formerly referred to as a contaminant).

Functionality: The set of performance criteria that the excipient is intended to meet when used in a formulation.

Genetically Modified Organism (GMO): Living organisms such as animals, plants, or microbes with an altered genetic makeup produced using a special set of technologies.

Impurity: A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.

Impurity Profile: A description of the impurities present in the excipient.

Mass Balance: The sum of the quantifiable material present in the excipient.

Packaging: The container and its components that hold the excipient for transport to the user.

Physical Property: A quality parameter that can be measured solely by physical means.

Physiological Effect: Any effect on the normal health of the human body.

Process: The set of operating instructions describing how the excipient is to be synthesized, isolated, purified, packaged, etc.

Process Parameter: A measurable operating condition.

Process Step: An instruction to the BPE manufacturing personnel directing that an operation be done.

Process Validation: A documented program that provides a high degree of assurance that a specific process will consistently produce a result that will meet predetermined acceptance criteria.

Raw Material: Any substance used in the production of an excipient, excluding packaging materials.

Replacement In Kind: Manufacturing equipment that uses the same operating principles and is of similar construction or packaging components made with the same materials of construction and sealed in a similar manner.

Reprocessing: Introduction of previously processed material that did not conform to standards or specifications back into the process and with repetition of one or more necessary steps that are part of the normal manufacturing process.

Residual Solvent: An organic volatile chemical that is used or produced in the manufacture of excipients. The residual solvent is not completely removed by practical manufacturing techniques.

Scale: An increase or decrease in the batch size in batch processing or the throughput capability for continuous processing, whether or not different equipment is used.

Significant Change: A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

Site: A defined location of the equipment in which the excipient is manufactured. It may be within a larger facility. A change in site may be to a different part of the existing facility but in a different operational area or may be to a remote facility, including a contract manufacturer.

Solvent: A vehicle, other than water, used in the synthesis of the product that remains chemically unchanged.

Specification: The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

Statistical Quality Control (SQC): The plotting of sequential test results to show their variation relative to the specification range and their normal variation.

Transmissible Spongiform Encephalopathy (TSE): Any agent that causes a symptomatic illness in animals or humans akin to BSE and variant Creutzfeldt-Jakob disease (vCJD), e.g., scrapie in sheep.

APPENDICES

Appendix 1: Change Levels

For guidance, examples of changes that typically would be classified into the three change levels are provided.

LEVEL 1

1. A processing parameter changed to a new set point that is within the process validation.
2. Use of alternate equipment that is listed as an alternate in a regulatory document (i.e., Drug Master File).
3. Use of equipment that is a replacement in kind. This is typically new equipment that uses the same operating principles as the equipment replaced.
4. Revision to a specification for one of the excipient's raw materials that involves more stringent quality or conformance to additional pharmacopeias.
5. Addition of a test parameter or tightening of an existing parameter to an excipient specification that is used for informational purposes only. This is not used for quality improvement or control purposes.
6. Improved environmental control to prevent cross-contamination of the excipient. An example of this is an improved packaging room or additional segregation of manufacturing equipment.

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LEVEL 2

1. A processing parameter changed to a new set point that is outside of the process validation.
2. A site change returning the manufacture of an excipient to a site previously used for this purpose more than 1 year ago.
3. Process control that is outside the normal limits of variability. An example of this is new process control equipment for control of excipient properties not previously controlled that create process adjustments.
4. A change in the handling, storage, or delivery of the excipient. An example of a handling change is the movement of a powder with new powder-conveying equipment. The storage of the excipient in bulk versus the shipping container is illustrative of a change in storage. The delivery of the excipient in temperature-controlled trucks versus uncontrolled trucks exemplifies a change in delivery but not vice versa.
5. A change in container size or shape.

LEVEL 3

1. Addition or removal of a chemical entity from the manufacturing process. An example would be the addition or removal of a preservative agent, buffering agent, stabilizer, or catalyst.
2. Manufacture at a new site never used for this purpose.
3. Revision to a sales specification made for the purpose of improving the quality of the excipient either through improved process control or lot selection.
4. Use of a new package, such as a drum of a different construction (i.e., plastic versus steel).
5. Revision of the product label.
6. Revision of the tamper-evident seal.
7. A change to the stated shelf life or retest interval.

Appendix 2: Decision Tree

A decision tree has been developed to aid in classifying the change into levels. The diagram begins with the proposed change and guides the BPE maker to an indication of the likelihood that the change will affect the excipient user. The decision tree classifies the types of change that occur in excipient manufacture as involving the site of manufacture, the processing steps, packaging, and testing and quality control.

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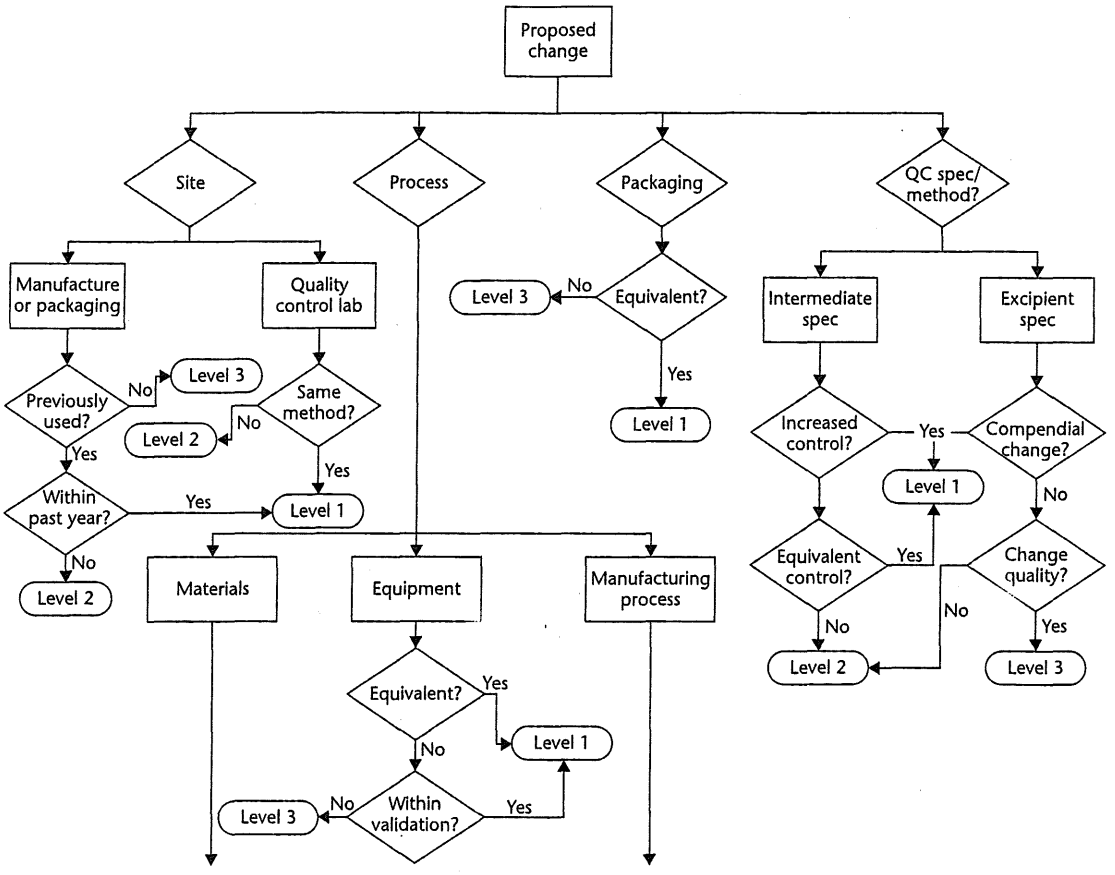
Appendix 2: Decision tree



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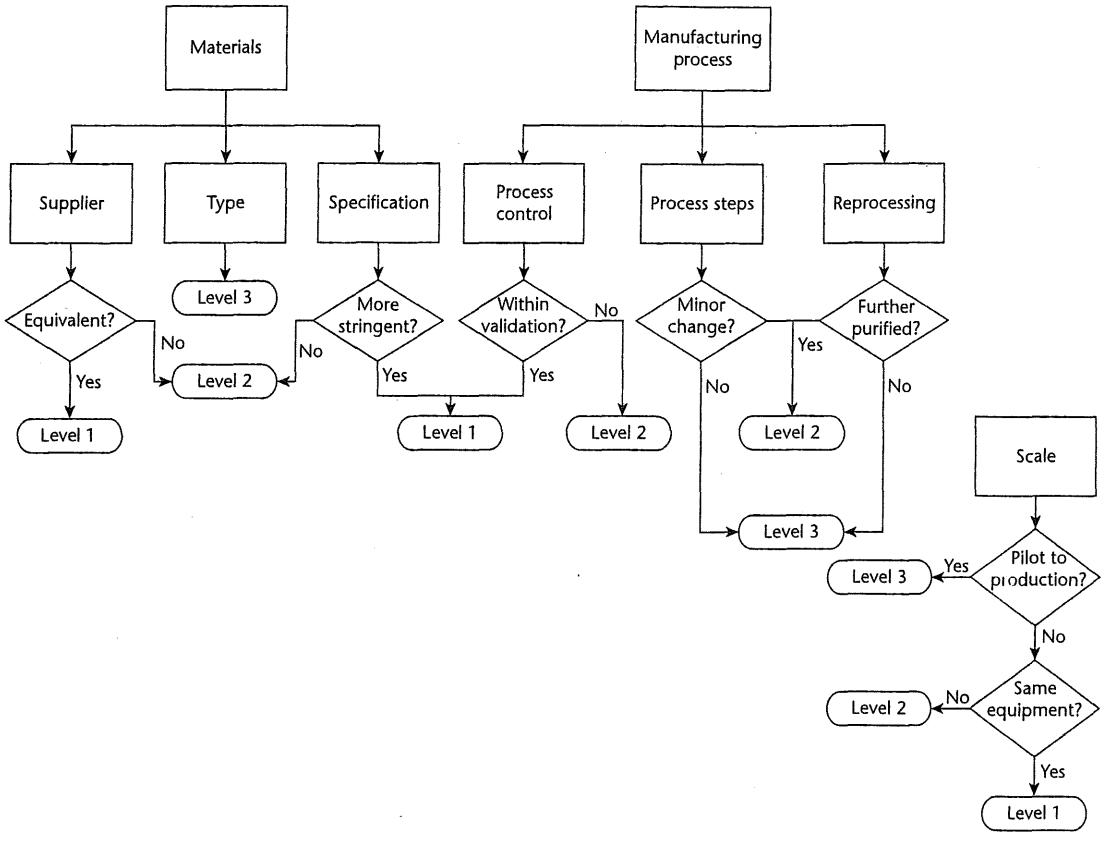
Appendix 2 : Decision tree (continued)



Appendix 3: Impurity Profile

DEFINITION OF IMPURITY PROFILE

The impurity profile of an excipient may be defined as a description of the impurities present in a typical lot of excipient produced by a given manufacturing process. The impurity profile includes the identity of each major impurity or an appropriate qualitative description, such as peak retention time (if unidentified), the quantity of impurity observed expressed as a range, and the classification, as discussed below, of each identified impurity. Excipients frequently function because they are not "pure". That is to say that often there are concomitant components that are necessary for the correct functioning of the excipient. These essential concomitant components should not be considered as part of the impurity profile but should be evaluated separately, if possible.

The composition of the impurity profile is dependent upon such variables as the raw materials, solvents, reagents, catalysts, and manufacturing process used in the excipient's manufacture. Foreign substances, such as manufacturing aids that can be present in the excipient, should be controlled to a level that is unobjectionable.[5]

It is recognized that the presence of essential concomitant components is important to the performance of the excipient in the drug product. Therefore, the presence of these essential concomitant components in the excipient should neither be construed as being undesirable nor be confused with the presence of foreign substances or impurities.

It should be noted that in some excipients, water may be an essential concomitant component, necessary to achieve the desired functionality. For other excipients, water may be included in the impurity profile, if appropriate, and should be classified as an inorganic impurity in such circumstances.

USE OF THE IMPURITY PROFILE

The impurity profile, as used in this chapter, is meant to help determine the significance of a change. Impurities should be profiled by the excipient manufacturer if possible. This may be accomplished through knowledge of the starting materials and manufacturing process and subsequent application of validated analytical testing to provide a qualitative and/or quantitative result of the impurity profile.

[5] Current USP General Notices.

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PROCEDURE FOR DEVELOPMENT OF AN IMPURITY PROFILE

Because of the diverse nature of substances that may be incorporated as pharmaceutical excipients, including highly complex mixtures from animal, botanical, mineral, and/or synthetic sources, differing approaches to characterizing their properties may be required. It is recognized that the development of an impurity profile may not be technically feasible for certain excipients. In such cases, the manufacturer should document what method is being used to monitor the excipient for the effect of changes as noted in this chapter in *Evaluation Criteria* and *Determination of Significance* in *Significant Change*.

CLASSIFICATION OF IMPURITIES

Excipient impurities are classified as follows.

Organic Impurities: Any organic material that arises during the manufacturing process that is not listed as the intended excipient in the monograph or specification and is not a concomitant component or foreign substance. This may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts.

Inorganic Impurities: Any inorganic material that arises during the manufacturing process that is not listed as the intended excipient in the monograph or specification and is not a concomitant component or foreign substance. This may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts.

Residual Solvents: Solvents resulting from the incomplete removal of organic or inorganic liquids, regardless of the source. See *Residual Solvents* ⟨467⟩ for details. Note that the limits specified apply to the drug product as considered in *Option 2* and to the excipient as in *Option 1*. It should be noted that a residual solvent can also be classified as a concomitant component but still must be considered.

IMPURITY PROFILE

The characterization of the impurity profile of an excipient should be attempted by the manufacturer, where possible, by taking into account the manufacturing process and potential impurities anticipated as a consequence. A sensible approach includes control of all impurities that have known toxicological characteristics. The limits of these impurities may be based upon the usage of the drug product when so informed by the user and should comply with the requirements of ICH Q3B(R) *Impurities in Drug Products* and of *Residual Solvents* ⟨467⟩.

For the purpose of developing an impurity profile, excipients may be classified as those where purity can be directly measured and those where purity cannot be directly measured. Examples of the former are excipients whose monograph or specification includes a requirement for purity. Polymers or derivatives of naturally occurring products are often examples of excipients where purity cannot be directly measured.

The material to be used for the development of the impurity profile should be sampled using the same sampling technique and sampling point in the manufacturing process as the sample taken for use in the quality control release of the lot.

EXCIPIENTS FOR WHICH PURITY CAN BE MEASURED

A mass balance is desirable, but it is recognized that a mass balance of 100% cannot generally be achieved because of the inherent limitation in accuracy and precision of the available tests, as well as the possible lack of suitable tests for some components. Mass balance of the excipient composition should be computed through the addition of the organic impurities, inorganic impurities, residual solvents, and excipient. If there are measurable essential concomitant components, they should be included with the excipient for purposes of this calculation. The purpose of calculating the mass balance is to estimate the amount of material not measured in the impurity profile. The excipient manufacturer should include in the report of the development of the impurity profile the mass balance achieved and what are thought to be the components not fully quantified.

Organic Impurities: Identify each impurity at or greater than 0.10% using appropriate analytical techniques. If organic impurities cannot be identified, a qualitative description, such as chromatographic retention time, should be assigned for all impurities at or greater than 0.10%. If direct measurement of organic impurities is not possible, total *Organic Impurities* can be reported as:

$$100 - (\text{Inorganic Impurities} + \text{Residual Solvents} + \text{Excipient})$$

Inorganic Impurities: Identify each impurity at or above 0.10% using appropriate analytical techniques. If direct measurement of inorganic impurities is not possible, total *Inorganic Impurities* may be estimated as:

$$100 - (\text{Organic Impurities} + \text{Residual Solvents} + \text{Excipient})$$

Residual Solvents: Report the solvents present by classification (see *Residual Solvents* ⟨467⟩) and level.

EXCIPIENTS FOR WHICH PURITY CANNOT BE MEASURED

Although a mass balance of the excipient composition of 100% is desirable, it is recognized that this goal is often technically difficult, if not impossible, to achieve. Therefore, manufacturers should include reports of the development of the impurity profile, the mass balance achieved, and what are thought to be the components not otherwise quantified.

For excipients produced by continuous chemical processing, it may not be possible to calculate a chemical mass balance, only an overall process balance.

Where direct measurement of the excipient purity is not feasible, techniques should be used to provide an estimate of excipient purity. This information is then applied in the equations listed above under *Excipients for Which Purity Can Be Measured*.

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DOCUMENTATION

The excipient supplier should develop documentation to support the development of an impurity profile. This documentation can be compiled in various ways by the supplier so that it can be retrieved to support the impurity profile. Documentation of an excipient impurity profile should include the following information:

1. Sampling plan
2. Analytical test methods
3. Identity and quantity of each component of the excipient, including both the excipient components and identified impurities
4. Discussion of the uncertainty in the measurement of each component of the excipient and impurity
5. Discussion of the mass balance

⟨1197⟩ GOOD DISTRIBUTION PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

SECTION 1. INTRODUCTION AND SCOPE

1.1 Introduction

Excipients are used in virtually all drug products and are essential to product performance and quality. Typically, excipients are manufactured and supplied so that they comply with compendial standards. The pharmaceutical excipient supply chain participants include manufacturers, distributors, brokers, suppliers, traders, transporters, forwarding agents, and repackagers. The quality of pharmaceutical excipients is affected by inadequate control of activities including distribution, packaging, repackaging, labeling, and storage. Improper or inadequately controlled trade and distribution practices can pose a significant risk to the quality of pharmaceutical excipients and can increase the risk of contamination, cross-contamination, adulteration, mix-ups, degradation, or changes in physical or chemical properties. To maintain the original and intended quality, all participants in the excipient supply chain should carry out their activities according to appropriate standards for good trade and distribution practices as discussed in this chapter.

[NOTE—The Appendix consists of definitions and acronyms.]

1.2 Scope

This general information chapter provides recommendations for those activities and practices that ensure good trade and distribution practices for pharmaceutical excipients in order to ensure their intended quality. These activities and practices include quality management, organization, documentation, premises, storage, equipment, stability, prevention of adulteration, importation, packaging, repackaging, labeling, dispatch, transport, returned goods, and compounding practices. In addition, personnel, authenticity of data, expiration dating, retesting, complaints and recalls, handling of nonconforming materials, internal/external/third-party audits, quality agreements, shelf life, traceability, economically motivated adulteration, and conformance to compendial monographs are included. The procedures outlined here are applicable to all persons and manufacturers involved in the handling of pharmaceutical excipients and apply to every step in the supply chain. This chapter covers all materials designated as, or intended for use as pharmaceutical excipients, beginning with the point in the manufacturing process at which the final excipient is designated for pharmaceutical use.

1.3 General Considerations

Manufacturers, distributors, users, regulators, and consumers expect pharmaceutical excipients to be manufactured, packed, stored, and transported in a manner that does not compromise their suitability for use in medicinal products for human or veterinary use. Because they are components of drug products, excipients are drugs within the meaning of the U.S. Federal Food, Drug, and Cosmetic Act (FD&C Act), and thus the U.S. Food and Drug Administration (FDA) definition of adulteration applies when an excipient is not fit for its intended use.

Excipients are a diverse group of materials. They can be of animal, mineral, synthetic, or vegetable origin, and they include materials that are solids, liquids, or gases. Excipients can be packed and transported in container sizes ranging from a few grams to a railroad tank car.

Because of their diverse nature and the number of ways in which excipients can be transported from the manufacturing site through the supply chain to the ultimate site of use, this general information chapter cannot provide exhaustive detail for specific materials and modes of transport. Rather, this chapter provides general guidance about what is expected of those people and organizations involved in the supply and distribution of pharmaceutical excipients intended for use in the manufacture of pharmaceutical finished products. Hence, there are instances when *USP–NF* chapters *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩, *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩, and *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩ provide a more detailed guide about what is expected in these specific areas.

Excipients also are used in a variety of industries. Although most drug substances typically are made exclusively for use in pharmaceutical finished products, the pharmaceutical use of an excipient may be only a small fraction of the total use of the

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material across all industries. This complicates the regulation of both the manufacture and the supply of pharmaceutical excipients. Excipients often are manufactured outside the United States, which further complicates the regulation of the manufacture and the supply of pharmaceutical excipients. Thus, all stages in the supply chain for the pharmaceutical excipient require transparency and proper flow of the necessary information regarding the excipient shipment. In addition, to ensure compliance with this chapter, suppliers of pharmaceutical excipients must follow all applicable national, regional, and local laws and regulations.

1.4 Pharmaceutical-Grade Excipients

Pharmaceutical excipients must be prepared according to the recognized principles of good manufacturing practices (GMPs) using ingredients that comply with specifications designed to ensure that the resulting substances meet the requirements of the compendial monograph (see *General Notices, 3.10. Applicability of Standards* and chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

USP or *NF* standards apply to any excipient marketed in the United States that is recognized in the compendium and is intended or labeled for use as an ingredient in a pharmaceutical product. The applicable standard applies to such articles whether or not the added designation *"USP"* or *"NF"* is used (see *General Notices, 3.10.10. Applicability of Standards to Drug Products, Drug Substances, and Excipients*). An ingredient may include the designation *"USP"* or *"NF"* in conjunction with its official title or elsewhere on the label only when a monograph is provided in the compendium and the article complies with the monograph standards and other applicable standards in the compendium including, but not limited to, the principles of GMP manufacture (see *General Notices, 3.20. Indicating Conformance*).

When *USP*- or *NF*-grade excipients are unavailable, manufacturers should first explore the use of materials which claim to comply with other pharmacopeias (e.g. EP, JP). If unavailable, pharmaceutical manufacturers should then consider appropriate alternatives (e.g. food-grade ingredients), provided such materials are suitable for the intended use. If a pharmacopeial grade is not used, a written justification should be available. The pharmaceutical manufacturer/user is responsible for the development and confirmation of suitable quality tests, procedures, and attributes to ensure that the material is appropriate for its intended use and that manufacturing is carried out under GMPs or a quality management system that demonstrates the same level of assurance of quality as that provided in *USP* (see ⟨1078⟩). It is an unacceptable practice to upgrade technical- or industrial-grade material to pharmaceutical-grade quality based only on analytical results that show compliance with the requirements of a pharmacopeial monograph.

1.5 Authenticity of Data

In the United States, the responsibility for the quality of the components of a finished pharmaceutical product lies with the organization that guarantees the quality of the finished pharmaceutical product. Thus, an important consideration in the purchase and supply of a pharmaceutical excipient is confirmation that the material is what it purports to be, that it meets specifications, that it was manufactured under applicable GMPs, that it has not been tampered with in any way before arriving at the site of intended use, that the appearance of the containers and other attributes of the shipment are comparable to those of previously received shipments of the same excipient and grade from the same supplier, and that it is fit for its intended use. Certain paperwork should accompany all shipments of pharmaceutical excipients. This paperwork should include a bona fide and legible copy of a Certificate of Analysis (COA) (see *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩).

When they receive a COA, manufacturers should take appropriate steps to verify the authenticity of the COA and the data contained therein. This has become particularly important in recent years because of instances of adulteration of excipients intended for use in the manufacture of pharmaceutical products. Steps to verify the authenticity of the COA should be taken at all stages in the supply chain.

Data on the COA can be verified in a number of ways, but the excipient user is responsible for confirming that the data are authentic by means of periodic verification of compliance with established specifications as stated in 21 Code of Federal Regulations Part 211 (21 CFR 211; see *Current Good Manufacturing Practice For Finished Pharmaceuticals*, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=211, http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM095852.txt). In addition, other documents such as dispatch notes from previous stages in the supply chain can provide further evidence of the pedigree of the excipient shipment. Such documents are termed "pedigree documents".[1]

This chapter may present additional challenges for certain excipient users, e.g., compounding pharmacies. However, those who compound still are obliged to take all reasonable steps to verify that excipients they receive are fit for their intended use. Part of this verification can include an examination of pedigree documents and a signed certificate of conformance (COC) from the suppliers. Information contained in the *USP–NF* monograph's labeling requirements, FDA's Inactive Ingredient Database, and the CFR provide specific information about the excipient's permitted use in FDA-regulated products. All purchasers of pharmaceutical excipients should establish written procedures for the verification of data and verification that the excipient is fit for its intended purpose.

[1] IPEC. The IPEC Excipient Pedigree White Paper. Arlington, VA: IPEC; ND. Available at: http://ipecamericas.org/sites/default/files/Excipient_Pedigree.pdf (Accessed July 6, 2011).

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SECTION 2: QUALITY, ORGANIZATION, AND DOCUMENTATION

2.1 Quality Management

A Quality Management System (QMS) is a tool by which all parties involved in the excipient supply chain maintain the quality of the excipient. A documented quality policy is the cornerstone of the QMS and formally describes the company's overall philosophy with regard to quality as authorized by top or senior management. Additionally, an appropriate QMS should include:

- An organizational structure capable of supporting the elements of the quality policy
- Documented procedures and relevant records that demonstrate that a product will meet established quality criteria. This is commonly known as quality assurance (QA)
- Established procedures for approving suppliers of starting materials and verifying that they continue to meet agreed-upon requirements
- A material-release testing procedure to confirm the quality of excipients for their intended purpose(s)

Excipient manufacturers and suppliers should prepare a Quality Manual. The Quality Manual describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes (see *Section 2.3 Documentation Requirements*). In implementing the QMS, companies must ensure that adequate qualified personnel are available to carry out the actions called for in the QMS and must avoid giving any one individual such extensive responsibilities that quality could be at risk.

COC to quality systems such as applicable International Organization for Standardization (ISO) guides or hazard analysis and critical control point (HACCP) analyses are not mandatory but provide assurance that products are produced and handled appropriately. However, certification to these quality systems should not be viewed as a substitute for the information contained in this chapter. In addition, internal audits should be conducted at regular intervals to confirm compliance with GMP (as applicable) and good distribution practices (GDP), and manufacturers should seek opportunities for improvement (see *Section 2.7 Audits: Internal, External, and Third-Party*).

All parties involved in the excipient supply chain share responsibility for the quality and safety of pharmaceutical excipients. These responsibilities should be delineated in a quality agreement between parties in the supply chain (see *Section 2.9 Quality Agreements*). All parties and their activities in the supply chain should be documented, and records should be maintained according to written procedures that ensure the traceability of all products acquired and distributed. All members of the supply chain have an obligation to protect excipients in their custody from deliberate economically motivated adulteration or deliberate introduction of foreign materials that could compromise the quality or performance of the excipient or adversely affect human or animal health.

2.2 Organization and Personnel

The organizational structure should be adequate and sufficiently staffed, and workers should be appropriately authorized for the activities they conduct. An organizational chart should delineate the responsibilities and interrelationships of personnel. Management ultimately is responsible for implementation of GDPs and ongoing verification that the QMS is maintaining the intended excipient quality.

Individuals within the company should have clearly defined responsibilities that are documented in writing. All individuals should understand their responsibilities and should be suitably qualified to perform their assigned duties. Their qualifications should be assessed for adequacy for their responsibilities and should be documented. Qualifications can include a combination of formal education, training, and experience. This also extends to any contracted service providers. Procedures should be in place to ensure that permanent, temporary, and contract employees minimize the possibility that unauthorized individuals will handle products.

An employee at each supply chain site should be designated and given the authority and responsibility for the implementation and maintenance of the QMS. The designated employee should have sufficient authority, qualifications, and resources to perform this function, as well as to identify and correct deviations from the QMS. Management and other personnel must not be subject to conflicts of interest or other pressures that could have an adverse effect on their ability to perform their duties related to product quality.

Staff should be aware of the principles of GDP included in this chapter and should receive regular, on-going training relevant to their responsibilities and to general quality principles. All training should be conducted according to a written training plan, and records of this training should be maintained. Personnel who have special duties such as handling hazardous materials or supervising activities required by local legislation may require additional training, including specific hazard management. Effectiveness of training should be verified regularly.

Personnel working with open product must understand and maintain good hygiene, health, and sanitation practices. Staff should use appropriate, nonshedding, protective apparel that will protect the product from the sampler as well as the sampler from the hazards of the product. Established procedures should eliminate the potential for product contamination by personal items such as jewelry, food, drink, or tobacco products. Written procedures that address hygiene, health, sanitation, and protective apparel should be in place.

Each supply channel party should have in place disciplinary procedures to address situations when personnel involved in the handling of products are suspected of or are implicated in inappropriate or illegal activities.

Some quality-related duties may be contracted to third parties, persons, or entities outside of the direct employ of the supplier. The delegation of these activities should be documented in a quality agreement or contract with the third party, and the organization should confirm compliance with the principles of GDP by conducting periodic on-site audits of these third parties. Delegation to a third party does not remove the organization's overall responsibilities for these activities.

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2.3 Documentation Requirements

2.3.1 GENERAL

Organizations should have in place a system to control documents and data that relate to the requirements of the QMS.

2.3.2 QUALITY MANUAL

Organizations also should maintain a quality manual that describes the QMS, the quality policy, and the company's commitment to applying the appropriate GDP and quality management standards contained in this chapter. This manual should include the scope of the QMS, reference(s) to supporting procedures, and a description of the interaction between quality management processes.

2.3.3 DOCUMENT CONTROL

Procedures for the identification, collection, indexing, filing, storage, withdrawal, archiving, maintenance, and disposition of controlled documents, including documents of external origin that are part of the QMS, should be established and maintained. Procedures used for the handling and distribution of excipients should be documented, implemented, and maintained. In addition, organizations should establish formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is used throughout the operational areas and that previous revisions of documents have been removed or withdrawn.

Designated qualified personnel should review documents and subsequent changes to the documents before the latter are issued to the appropriate areas. Documents that influence product quality should be reviewed and approved by the quality unit. Controlled documents may include a unique identifier, date of issue, and revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. The reasons for changes and the implementation date should be documented.

Electronic documentation should meet the requirements stated above for the document control system. If electronic signatures are used, they should be controlled to provide security equivalent to that given by a hand-written signature. Electronic documents and signatures also may need to satisfy local regulatory requirements.

2.3.4 CONTROL OF RECORDS AND DATA

Procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records and data should be established and maintained. Records and data should be maintained to demonstrate achievement of the required quality and the effective operation of the QMS. Records and data should be legible and clearly linked with the product or process involved. Pertinent third-party quality data also should be an element of these records.

Entries in records and data should be clear and indelible and should be made directly after the person performs the activity and then should be signed and dated by the person who made the entry. Corrections to entries should be signed and dated, leaving the original entry legible and with an explanation for the change, especially if this may not be obvious to subsequent reviewers.

Records and data should be kept for a defined period that is appropriate for the excipient, its use, and its retest or re-evaluation date. Records and data should be stored and maintained in such a manner that they are readily retrievable and in facilities that provide a suitable environment to minimize deterioration or damage. Electronic records and automated data-capture systems should meet the requirements for controlled records and data as stated above.

2.3.5 CHANGE CONTROL

Procedures to evaluate and approve all changes, including evaluating the impact of the change on the quality of the excipient, should be established and maintained, for example, changes to the following:

- Authorized excipient manufacturer or packaging material supplier
- Manufacturing or packaging sites
- Excipient or packaging material specifications
- Test methods and laboratory
- Repackaging, labeling, and storage equipment
- Analytical equipment
- Repackaging, labeling, and storage processes
- Process and equipment changes at the original excipient manufacturer's site (see *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩)

An independent QA group should have the responsibility and authority for the final approval of any changes. The QA group may be part of another operational unit such as regulatory affairs or research and development.

Customers and, if applicable, regulatory authorities (e.g., those responsible for drug master files or certificates of suitability to the *European Pharmacopoeia*) should be notified of significant changes to established production and process control procedures that could affect excipient quality. The original manufacturer and downstream intermediaries (distributors and traders) should have excipient change control agreements in place defining the extent of notification by the original manufacturer in case of a change as described above. Each of the handling parties within the supply chain should have change control agreements to ensure that changes from the original excipient manufacturer are communicated to the end user. This change control agreement is part of the overall contractual agreements between the parties.

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2.4 Complaints and Deviations

Customer complaints and information about possible defects should be systematically documented and investigated based on a written procedure with assigned responsibilities that describes the action that will be taken and includes the criteria on which a decision to recall a product should be based. Investigations should be formally conducted and written up in a timely manner to establish if the complaint is justified, to identify the origin or reason for the complaint (e.g., the repackaging procedure, the original manufacturing process, etc.), to identify root cause(s), to define any initial and follow-up action(s), and the method of communication (e.g., to the customer, original excipient manufacturer, authorities, etc.). Complaint records should be retained and regularly evaluated for trends, frequency, and criticality in order to identify possibly needed corrective or preventive actions.

Investigations should identify whether the reported defect is limited to a single batch of material or if other batches must be investigated. If additional batches are implicated, they should be identified and labeled accordingly (e.g., "under quarantine"). As necessary, appropriate follow-up action, possibly including a recall (as outlined in *Section 2.5 Recalls*), should be taken after investigation and evaluation of the complaint. Confirmed serious problems related to product quality (e.g., faulty manufacturing, packaging, or product deterioration) should be communicated upstream to the manufacturer and downstream to customer(s) in case they received material with the same batch number. A similar process should be implemented for the handling of deviations and product defects not identified by a customer complaint.

2.5 Recalls

Those involved in the excipient supply chain should have a system for recalling promptly and effectively any materials known or suspected to be defective. Entities involved in the supply chain should implement written procedures to manage excipient recall (retrieval) in a timely manner. The procedures should:

- Describe how the process of recall (retrieval) should be managed based on the risk involved
- Describe a decision-making process with defined responsibilities
- Define the functions involved in the process (e.g., QA, sales, logistics, senior management, competent authorities, etc.)
- Define the communication process and documentation to parties within the supply channel as well as to regulatory authorities
- Define the steps needed to retrieve the material

If the original excipient manufacturer does not initiate a recall, it should be informed of the recall. Entities in the supply chain should have written procedures for the organization of any recall activity, and these should be regularly checked and updated. All recalled materials should be stored in a secure, segregated (quarantined) area while their disposition is decided. In the event of serious or potentially life-threatening situations, all customers and competent authorities in all countries to which an excipient potentially was distributed should be promptly informed of any intention to recall the excipient. All records should be readily available to the designated person(s) responsible for recalls. These records should contain sufficient information about materials supplied to customers (including exported materials). At regular intervals, QA groups in supply-chain organizations should evaluate the effectiveness of recall arrangements.

2.6 Handling of Nonconforming Materials

Nonconforming materials should be handled in accordance with a procedure that will prevent their inadvertent introduction or reintroduction into the market. They should be stored separately, either physically separated or under electronic control, to prevent their inadvertent introduction into commerce. Firms that conduct recalls should maintain records covering all activities, including destruction, disposal, return, and reclassification, and should perform an investigation to establish whether any other batches also are affected. They should document the investigation and actions taken to prevent recurrence of the problem. As necessary, firms should take corrective measures. Procedures should exist for the evaluation and subsequent disposition of nonconforming products, and the disposition of the material, including downgrading to other suitable purposes, should be documented. Nonconforming materials should never be blended with materials that comply with specifications.

2.7 Audits: Internal, External, and Third-Party

To verify compliance with the principles of GDP for pharmaceutical excipients, firms in the excipient supply chain should perform regularly scheduled internal audits in accordance with approved procedures. Firms should document audit findings and corrective actions and ensure that they are brought to the attention of responsible management. Accepted corrective actions should be completed in a timely and effective manner and should be conducted by designated, qualified individuals. Qualified individuals may be employees of the company, but they must be sufficiently removed from the function under audit so that their independence is not compromised.

Firms should perform external audits in accordance with approved procedures and schedules to assess the capability of suppliers to meet requirements for a product or service, as specified. A response to a questionnaire may be considered in the auditing process but generally does not take the place of on-site inspections and should not be considered a substitute when an audit is required. Independent auditing organizations can perform third-party audits to determine the level of compliance or conformance to specified standards and regulations (e.g., GMP, GDP, and ISO).

2.8 Contract Activities

Any GDP-related activity that is delegated to another party should be agreed upon in writing in an approved contract with clearly defined responsibilities. The contract should clearly establish which party is responsible for each applicable quality

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activity. Before entering into an agreement, the contract giver should evaluate the proposed contract acceptor's compliance with GDP as described in this general chapter. The evaluation should include an initial on-site audit of the contract acceptor's premises and quality system, giving special consideration to the prevention of cross-contamination and maintaining traceability. The contract should also include the responsibilities of the contract giver for measures to avoid the entrance of counterfeit or adulterated materials into the distribution chain.

There should be no gaps or unexplained lapses in the application of GDP. The contract acceptor should conduct periodic on-site auditing of contracted distribution activities with regard to the application of GDP by the contract giver. Subcontracting may be permissible under certain conditions, subject to approval by the original contract giver, especially for activities such as sampling, analysis, repacking, and labeling. If subcontracting occurs, the subcontractor should conform to the same GDP standards as the primary contract giver. The subcontractor also should permit an on-site audit by the contract acceptor's quality unit or its designee.[2, 3]

2.9 Quality Agreements

Quality agreements are legally binding and are mutually negotiated between parties involved in the supply chain for pharmaceutical excipients. The quality agreement identifies who is responsible for certain quality activities and how quality issues will be resolved between the parties. Although they are intended to address the parties' quality commitments, quality agreements are not designed to take the place of an audit.

Suppliers should have in place quality agreements between themselves and the parties with whom they do business. Original excipient manufacturers should have quality agreements in place with their direct customers and authorized distributors of their products. Distributors should also have agreements with end users and other parties in the supply chain to whom they supply products. All entities in the supply chain should fully understand which entity is responsible for the GDP-related activities (as outlined in this chapter) at each step in the supply chain.

Quality agreements should address the quality systems requirements, but they are not intended to list every element of the quality system. It is not necessary to reiterate agreement on every point of the quality system when the parties state general agreement on the applicable quality standard. Quality responsibilities included in a quality agreement should be those that may require action by one or both parties to the agreement.

A key element that must be defined in the quality agreement is the communication pathways and timing for quality events. Parties must be clear about their responsibility for notifying the next party in the supply channel and for notifying the applicable regulatory authorities in the case of a significant quality event. Many times a decision about who should notify the regulatory authority is a collaborative effort between the parties. Depending on the issue's impact, the timing of these notifications relative to the time of the incident should be specified within the quality agreement.

Both parties to the agreement are responsible for ensuring that the quality agreement is maintained as an accurate document throughout the life of the business relationship. Revisions to this document may be needed as regulatory requirements change, new products are supplied, or a new material risk arises. The parties should maintain a history of the revisions to the quality agreement.

SECTION 3: PREMISES, STORAGE, REPACKAGING, AND STABILITY

3.1 Buildings and Facilities

Organizations should establish operating procedures for the use of buildings and facilities, including the areas discussed below, and firms should consider protective measures to ensure the security of the grounds (e.g., fencing or perimeter walls).

The buildings and facilities used in the storage and handling of excipients should restrict access to allow entrance only by authorized persons to areas used for the manufacture, packaging, and holding. Organizations should take precautions to prevent unauthorized persons from entering limited-access areas. When the status of excipients requires protection from use (e.g., quarantine), organizations must have clearly marked limited-access controls in place, or they should use validated computerized systems to prevent material distribution before approved release.

Buildings should be of adequate size and capacity to allow the orderly flow of materials, proper storage and handling of materials, and appropriately controlled environmental conditions for the final dispatch of excipients into and out of the premises. Buildings should be maintained in a good state of repair. The construction materials must be easily cleanable and maintained, and buildings and facilities should be designed to prevent cross-contamination, product mix-ups, or the accumulation of filth or contaminating materials, particularly when excipients are exposed to the environment. Adequate storage space must be available for excipients that are highly sensitizing or toxic, and dedicated facilities may be necessary. Adequate procedures should be in place to ensure the cleaning, maintenance, and use of buildings and facilities.

Receiving and dispatch bays should be designed to protect the facilities and excipients during loading and unloading during adverse weather conditions. Incoming bay areas should be designed and equipped to allow containers to be cleaned before storage. A pest-control system should be in place to ensure that materials are protected from infestation by insects, rodents, animals, birds, or other vermin. There should be written procedures defining the adequate holding and storage of excipients, including pest-control processes. The pest-control materials must be safe and must be known not to cause contamination. Approved pesticides, insecticides, and rodenticides should be used and documented. Excipients that may contain

[2] WHO. *Good Trade and Distribution Practices for Pharmaceutical Starting Materials*. Geneva; WHO: Technical Report Series, No. 917, 2003, Annex 2. Available at: http://who.int/medicines/areas/quality_safety/quality_assurance/GoodtradeDistributionPracticesTRS917Annex2.pdf?ua=1 (Accessed March 2, 2015).

[3] WHO. *Finished Products: Good Distribution Practices for Pharmaceutical Products*. Geneva; WHO: Technical Report Series 957, 2010, Annex 5. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_957_eng.pdf (Accessed July 7, 2011).

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contamination must be controlled to prevent cross-contamination in holding areas or the spread of contamination to other areas of the facility.

3.2 Warehousing and Storage

Written procedures should describe the receipt, storage, dispatch, and other handling of excipients, as well as the security measures necessary to prevent theft of materials or the introduction of counterfeit or adulterated materials into the supply chain. Buildings should be adequately lighted and should have proper utilities for the intended activities. They should be dry and controlled to appropriate environmental conditions. Buildings and facilities should store excipients in the proper environmental conditions. Temperature-controlled and -monitored storage should be available as required for any building used for holding excipients.

Warehousing and storage conditions for excipients should comply with the monograph specifications, as reflected in the excipient's container label. When specific storage conditions are required for excipients (e.g., temperature and humidity control), they should be provided in a controlled manner, monitored (e.g., by an alarm system or manual control), and recorded. Any automated system(s) used to monitor the environmental conditions for areas where excipients are handled or stored must be validated. An approved document should indicate the location of each environmental monitoring device and the condition(s) it monitors. The locations for these devices or probes should reflect the extreme environmental conditions of the space as determined by an environmental mapping exercise. Excipients that present risks such as fire or explosion should be stored in safe, dedicated areas. Excipients that are sensitizing or toxic should be adequately and appropriately segregated, and warehouse and storage areas should be routinely cleaned, appropriately maintained, and free of pests.

Excipients should be stored in a manner that permits cleaning of the storage area and movement of materials. Pallets used to hold materials should not cause contamination, and required pallet quality and construction materials should be defined in writing. Pallets should be clean and in a good state of repair, and firms should appropriately track supplies to ensure adequate treatment of the wood materials. Wood pallets, if used, should comply with import requirements.

Organizations should have in place written procedures to ensure that the excipient will be supplied within its expiry or retest period and should have adequate controls to prevent the distribution of expired excipients. If no expiry date is applicable, the first-in-first-out principle should be used. Rejected excipients and other materials related to excipient quality (e.g., packaging components) should be so labeled or identified, and controls such as physical or electronic separation should prevent their use pending final disposition. During the warehousing or storage of excipients, any known broken or damaged containers should be withdrawn from usable stock, and the containers should be handled as rejected materials.

Materials quarantined pending a release decision should be labeled or identified (e.g., electronically) to prevent unauthorized use. These materials should be held from use, and written procedures should guide final disposition. There should be written procedures for the cleanup of any spillage to ensure complete removal of any risk of contamination.

3.2.1 ENVIRONMENTAL CONTROLS

When excipients require specific storage conditions to preserve their integrity and quality during the retest/re-evaluation or expiry interval, the storage conditions required should be stated on the label, labeling, or other literature, e.g., the Excipient Information Package[4] or COA. Distributors should follow the information and requirements for environmental controls provided by the manufacturer and should provide appropriate controls and monitoring to ensure adherence to the stated storage conditions with appropriate documentation. Distributors should also maintain records to indicate the excipient was stored according to the manufacturer's recommendations and should conduct regular assessments to confirm that designated conditions are met.

If the manufacturer does not indicate specific storage conditions, the distributor should ensure that proper storage conditions are maintained to protect the packaging and labeling. Uncontrolled warehousing conditions vary with geographical location, particularly with latitude. If the excipient is shipped to geographical locations that have storage conditions well outside the conditions used in the manufacturer's stability study or justification for the absence of special storage conditions, then additional studies may be required to show stability at the new conditions. A warehouse-monitoring program should be established if the effects of the new environmental conditions are not known.

Outdoor storage of excipients (for example, bulk materials, flammable materials, acids, or other corrosive substances) is acceptable provided the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, discharge ports have adequate protective closures, and the exteriors of moveable containers are adequately cleaned before opening and use.

3.3 Equipment

Equipment used in bulk transport, repackaging, labeling, testing, or storage of the excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation. Equipment should be verified before use to ensure that it is constructed, installed, and functioning as required for the excipient. When equipment is located outdoors, there should be suitable controls (e.g., closed systems or protective encasements) to minimize environmental risks to excipient quality.

When possible, dedicated equipment (e.g., bulk trucks, packaging equipment, storage tanks, pipework, hoses, and pumps) should be used in direct contact with the excipient. When nondedicated equipment is used in direct contact with the excipient, validated cleaning procedures should be applied. A restricted prior-cargo list should be supplied to transport companies in case non-dedicated bulk transport equipment is used. Quality-critical measuring equipment and balances for the handling and testing of the excipient should be of appropriate range and precision. Such equipment should be identified.

[4] IPEC. *The IPEC Excipient Information Package (EIP): Template and User Guide*. Arlington, VA: IPEC; 2013. Available at: http://ipecamericas.org/reference-center/document-depot (Accessed June 23, 2017).

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3.3.1 EQUIPMENT CONSTRUCTION

Equipment in contact with an excipient should be constructed so that contact surfaces are not reactive, additive, or absorptive and thus do not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, preferably should not come into contact with excipients and packaging materials. When contact is possible, distributors should use materials of suitable quality that will not affect product quality. The choice of such materials should be justified.

Equipment should be designed to minimize the possibility of contamination from the environment and direct operator contact during activities such as unloading bulk trucks, use of transfer hoses (particularly those used for transfer of excipients), sampling, repackaging, and cleaning. Distributors should consider the sanitary design of equipment in contact with excipients. They should assess the suitability and integrity of seals in order to minimize the risk of contamination. Piping should be appropriately labeled to indicate the content and direction of flow.

3.3.2 EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the repackaging, labeling, testing, or storage of the excipient. Distributors should maintain records (e.g., logs, computer databases, or other appropriate documentation) of quality-critical equipment use and maintenance. Defective equipment should be removed or appropriately labeled to avoid misuse.

Quality-critical measuring equipment and balances should be controlled on a scheduled basis. This control should include the following:

- Calibration of instruments or other appropriate verification at suitable intervals, according to an established documented program
- Establishment of the equipment's limits of accuracy and precision
- Provisions for remedial action in the event that accuracy or precision requirements are not met

Calibration standards should be traceable to recognized national or compendial standards as appropriate. Instruments and equipment that do not meet established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration or verification status of quality-critical equipment should be known to users and should be verifiable.

3.3.3 EQUIPMENT CLEANING

Cleaning equipment should be chosen and used so that it cannot be a source of contamination. Cleaning materials should be appropriate for the task, and their selection should be justified. Rotation of sanitizing and cleaning agents should be considered where appropriate. In order to avoid contamination with cleaning products or products previously processed in the equipment, written cleaning procedures should be established for equipment that comes in contact with the excipient. Cleaning procedures should contain sufficient detail to allow cleaning in a reproducible and effective manner. Cleaning and sanitation processes should be recorded, and evidence of their effectiveness should be provided, for example, by the following:

- Testing the final rinse after cleaning for residues of the previous product
- Checking the equipment after cleaning for residues of the previous product
- Testing each batch for residues of the previous product handled with the same equipment

3.4 Sampling, Repackaging, and Labeling

To minimize risks associated with repackaging and labeling, appropriate GMPs should be applied (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩). For completeness, certain key activities and the necessary precautions are discussed below.

3.4.1 BLENDING, REPACKAGING, AND LABELING

Operations such as combining sublots into a homogeneous batch, repackaging, or labeling are manufacturing processes, and therefore distributors should follow appropriate GMPs (see ⟨1078⟩):

- Processes whereby the excipient's packaging is opened and the excipient is exposed to the environment (for example, transferring excipient from one container to another, including from bulk equipment to storage tanks/silos or from storage tanks/silos into containers) are critical handling steps related to the integrity of the finished product. If only the secondary packaging is modified, operators should take appropriate care to maintain the integrity of the primary packaging and the excipient.
- Excipients may degrade because of exposure to the repackaging atmosphere (e.g., oxygen, humidity, light, and temperature).
- Excipients can be contaminated by foreign matter such as lubricants, cleaning materials, or other substances.
- Transparency to the customer that relabeling, with or without opening the original excipient manufacturer's packaging, has occurred is critical to representation of the product quality and suitability for use.
- Transparency to the customer of data sources listed on certification documentation (labeling) is critical to representation of the product quality and suitability for use.

3.4.2 REPACKAGING AND LABELING BATCHES

Staff in the excipient supply chain should give special attention to the following points:

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- All repackaging and labeling requirements should be defined in written procedures.
- Contamination, cross-contamination, and mix-ups should be avoided by the use of suitable equipment and cleaning procedures and with adequate labeling.
- Environmental conditions and repackaging procedures should be designed to avoid contamination and to maintain the integrity of the excipient during repackaging and labeling.
- Operators should consider the use of filtered air in the repackaging area if necessary for the product. The standard of filtration should be justified.
- Labels should be printed using a controlled process (see *Section 3.4.9 Repackaging and Labeling*).
- Personnel involved in repackaging processes should wear clean protective apparel such as head, face, hand, and arm coverings, as necessary, and should practice appropriate personal hygiene (e.g., hand disinfection following health requirements, health monitoring, and removal of jewelry). Personnel should be trained about special hygiene requirements, and this training should be documented.
- Repackaging areas should be cleaned and sanitized regularly.

Batch numbers should be assigned according to documented procedures. When staff assigns new batch numbers, they should ensure traceability to original batch numbers by proper documentation. Assigning one batch number to containers of different batches that comply with the same specification is an unacceptable practice (see also *Sections 3.4.3 Excipient Batch Homogeneity* and *3.4.4 Blended Excipients*).

- As part of the batch record, a copy of the information on the original labels should be retained (e.g., a photocopy). A sample of the new label should also be kept.
- All repackaging and labeling processes should be designed and carried out to avoid commingling, contamination, and mix-up and to ensure full traceability of the excipients back to the original excipient manufacturer and traceability downstream to the final customer. Responsible personnel should sufficiently record every completed step, along with the name of the operator and the date and time each step was completed, e.g., in the master batch manufacturing record, or by means of computerized systems.

3.4.3 EXCIPIENT BATCH HOMOGENEITY

Mixing to form a homogeneous batch is a manufacturing step and should be defined in a written procedure. A batch can be homogeneous only when conforming materials are thoroughly mixed. The conformity of each batch with its specification should be confirmed before it is added. Mixing should always be controlled, and homogeneity should be verified and documented (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩). Blending of batches or lots of excipients that individually do not conform to specifications with other lots that do conform (in an attempt to salvage or hide adulterated or expired material) is not an acceptable practice. Only excipients from the same manufacturing site received by a distributor and shown to conform to the same specifications can be mixed. The customer should be informed that the material supplied is a mixture of the manufacturer's batches.

3.4.4 BLENDED EXCIPIENTS

The blending process should be verified to ensure that it does not influence the quality of the excipient. The blended excipient should be tested to ensure conformance to the specification and to provide data for the COA (see *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩). Under certain circumstances and with appropriate controls, a COC can be used if the basis for the claim of conformity is traceable within the document. The blended batch referred to in the new certification document should be traceable to all the original certification documents and batch numbers (see ⟨1078⟩).

3.4.5 CERTIFICATES OF ANALYSIS

The original excipient manufacturer's COA should be retained and made available to the user on request. The batch referred to in the COA delivered to the end user should be traceable to the original excipient manufacturer's COA. Quality documents accompanying deliveries should be subject to an agreement between the distributor and the final customer. For retesting, analytical methods of the original excipient manufacturer or pharmacopeial methods should be applied. When other methods are applied, these should be agreed upon by both parties.

3.4.6 CONTAINER–CLOSURE SYSTEMS

For repackaged material, the repackager is responsible for justifying the shelf life and repackaging conditions. The original manufacturer and the distributer should share information and agree about repackaging conditions and primary packaging materials. They should establish primary container–closure system material and packaging configuration specifications, and they should develop a written procedure that clearly defines packaging for each individual excipient based on its stability.

If the same types of primary container–closure system and packaging configuration are used for repackaging, then the new container–closure system and packaging configuration should be equivalent to that used by the original excipient manufacturer. The repackager and distributor should consider exposure of the excipient to the repackaging environment, and both can rely on the manufacturer's stability evaluation and thus assign the same shelf life for the excipient.

When the repackager's primary container–closure system's packaging configuration differs significantly from that of the original manufacturer [e.g., in terms of desiccants, permeability of the protective barrier layer (which may be either the primary or secondary container–closure system), or the headspace], the repackager must demonstrate that the new system is adequate to protect the excipient from contamination and deterioration for the shelf life (retest or expiration period) defined by the excipient manufacturer. Otherwise, the shelf life defined by the manufacturer cannot be transferred to the repackaged material.

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The need for stability studies should be confirmed (see *Sections 3.4.14 Stability and Expiration Dates* and *3.5 Retesting and Shelf Life*).

The container–closure system for the pharmaceutical excipient should protect the material from the time of packaging until its final use by the drug product manufacturer. The container–closure system should be designed to help prevent theft or adulteration by counterfeiting.

Storage and handling procedures should protect containers and closures and minimize the risk of contamination, damage or deterioration, and mix-ups (e.g., between containers that have different specifications but are similar in appearance).

3.4.7 RETURNED AND REUSED CONTAINERS

Returned containers may have unknown residues from uses other than the intended one. Therefore, use of new containers is recommended for excipients. If containers are reused, a rationale for the extent of cleaning should be justified and documented for specific excipients and different types of containers. Repackagers should collect evidence that the quality of the material packed is not adversely affected by reuse of containers.

Distributors and customers should have an agreement defining the specific conditions for reuse (e.g., handling, sealing, and cleaning). If returnable excipient containers are reused, all previous labeling should be removed or obliterated.

3.4.8 ENVIRONMENTAL CONTROLS

Environmental controls should ensure that temperature, humidity, and cleanliness of air and equipment are appropriate to avoid any contamination to or deterioration of the excipient. The necessary environmental conditions for the repackaging of each excipient should be defined. Environmental control is a specialist subject, and experts should be consulted (see also *Section 2.6 Handling of Nonconforming Materials*).

3.4.9 REPACKAGING AND LABELING

Repackagers should implement procedures to ensure that the correct quantity of labels is printed and issued and that labels contain the necessary information. Sufficient crosschecks should be in place to ensure proper data transfer. Procedures should be in place to avoid mislabeling, and printing and use of labels should be restricted. All labeling operations (e.g., generating, printing, storage, use, and destruction) should be recorded. Labeled containers should be inspected, and surplus labels should be destroyed to avoid any misuse. If labels are not printed immediately before each specific labeling operation, the security of the label stock should be controlled, and access limitations should be defined. Repackaging and labeling facilities should be inspected immediately before use to ensure that all materials that are not required for the next repackaging operation have been removed.

3.4.10 REPACKAGED EXCIPIENTS—ACCOMPANYING DOCUMENTATION

Deliveries of repackaged excipients should be accompanied by information about the original manufacturing site (name and address) and repackaging and labeling sites. This information should be provided in the supplier certification documentation (e.g., COAs) or by other means (see *Section 4.8 Traceability*). The supplier should provide this information to the customer via official communications.

3.4.11 TESTING OF REPACKAGED EXCIPIENTS

Appropriate testing of repackaged excipients should be performed to demonstrate consistent excipient quality. Testing to the complete monograph may not be necessary, but the recipient should test defined key quality parameters that could be affected by the repackaging process. Recipients should consider the manufacturer's recommendations for key quality parameters, and until these tests have been performed the repackaged materials should be kept under quarantine and should be identified as quarantined material. The materials should comply with the defined specifications before they are released for distribution.

Excipient testing and release should be performed under the responsibility of the quality unit and should conform to written specifications and analytical test requirements. Repackagers should ensure that test data are recorded and that results are evaluated before release of the repackaged or transferred excipient.

The excipient cannot be upgraded as a result of any repackaging process. It is unacceptable to upgrade nonpharmaceutical grades to pharmaceutical grades on the basis of conforming analytical results, i.e., by testing to pharmacopeial standards. Pharmaceutical grades can be achieved only when the excipient is originally produced and subsequently processed in accordance with GMPs (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

3.4.12 OFFICIAL PHARMACOPEIAL METHODS FOR RETESTING

For control of key parameters during repackaging or full retesting of excipients, official pharmacopeial methods or methods validated against the pharmacopeial methods should be used. Otherwise, repackagers should use the original excipient manufacturer's analytical methods. The methods used should be listed on the COA accompanying the excipient or should be made available to the customer by other documents. These documents should also reference any contract laboratory that is used to perform analyses. The COA should clearly identify which tests have not been performed on the repackaged or transferred batch but have been taken from the original manufacturer's COA.

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3.4.13 SAMPLING

Excipient sampling must be done in a manner that prevents contamination, and dedicated sampling areas with adequate environmental controls are necessary. Areas for sampling should be designed to allow cleaning of the outside of the container before the container is opened. Adequate cleaning procedures should be in place for the sampling areas. Sampling tools should be dedicated to the sampling area and also to the specific material, or sampling tool cleaning must be validated to ensure no cross-contamination from the tool.

Any container opened for sampling should be marked with the date and name of the person who performs this operation. The amount of sample removed should be recorded.

If excipients are repackaged, processed, or packaged from bulk, retained samples representative of the excipient batch should be kept for at least one year after the expiration or re-evaluation date or for at least one year after distribution is complete, whichever is longer. The minimum sample size should be based on the amount required to perform at least two complete analyses. Sample storage conditions should prevent any contamination or deterioration and should comply with the label storage conditions (see general information chapter, *Bulk Powder Sampling Procedures* ⟨1097⟩).

3.4.14 STABILITY AND EXPIRATION DATES

Excipient stability and expiration dating of excipients are primarily the responsibility of the original manufacturer. Whenever the original manufacturer's packaging is opened, the repacker is responsible for providing evidence that the excipient manufacturer's stability and expiration dating are still applicable.

If a distributor transfers an excipient to another container or repackages it, stability and shelf life (retest or expiry period) should be taken into account. The type of container, primary packaging materials, barrier packaging materials, packaging configuration, environmental exposure during repackaging, and storage conditions at the repackaging site should also be taken into account when the shelf life (retest or expiry period) is defined. The recommended expiration date provided by the original excipient manufacturer should not be extended without demonstrating sufficient stability to justify extended shelf life (retest or expiry period). If shelf life is extended beyond the original manufacturer's recommendation, the type of packaging, storage conditions, and stability-indicating analytical data should be clearly defined, and the repacker assumes the primary responsibility for the extension.

If special storage conditions (e.g., inert gas overlay, protection from light, heat, moisture, etc.) are needed, the restrictions should be indicated on the new labeling (see *Section 3.5 Retesting and Shelf Life*).

3.5 Retesting and Shelf Life

The organization's stated shelf life or retest/re-evaluation interval should be maintained for the excipient. Expiration or shelf life dates indicate the period beyond which the excipient should not be used or distributed. Retest/re-evaluation intervals indicate the period beyond which the excipient must be evaluated to determine continuing acceptability for use. The expiration/shelf life date provided by the original excipient manufacturer should not be extended without documentation from the manufacturer demonstrating sufficient stability to justify an extended shelf life. Such documentation should specify the type of container and storage conditions necessary to make this claim, and the distributor should have documentation that the excipient was stored in the stated container and under the necessary conditions.

Excipients without expiration, retest, re-evaluation, or shelf life dates should be accepted for use only if the manufacturing date can be confirmed and only if the excipient has been held and shipped under conditions that conform to the appropriate standards of GMP or GDP. Distribution of the excipient beyond the retest/re-evaluation period should be done only in consultation with the manufacturer and with the consent of the purchaser or recipient. If the distributor has the capabilities for sampling and performing the manufacturer's specified evaluation, then the distributor can perform the assessment. Sampled lots should be placed under quarantine to prevent shipping during the evaluation.

Distributors who do have capabilities for sampling according to the manufacturer's instructions but do not have testing or evaluation capabilities should send the samples to the manufacturer or a qualified third-party laboratory for retesting/re-evaluation. Excipient lots that conform to the manufacturer's criteria can be released from quarantine, and the distributor's supporting evaluation data should accompany the original excipient manufacturer's data to indicate the excipient's acceptability for use. If the distributor does not have the capability to sample or evaluate the excipient, it should not be shipped to customers beyond the end of the retest/re-evaluation interval. The excipient or a representative sample of the excipient can be returned to the manufacturer or a third party for retesting/re-evaluation. The excipient can be held by the distributor pending further results obtained from the representative sample.

If an excipient is transferred to another container or is repackaged by the distributor, the latter must conduct an assessment of the stability of the excipient to determine if the original excipient manufacturer's information can be carried forward. If the distributor uses the same type of packaging material that provides the same packaged environment (headspace, surface area, closure tightness, etc.) as that used by the original manufacturer and if the transfer or repackaging is performed in a manner that protects the excipient from adverse environmental effects that could affect the stability, then the original excipient manufacturer's shelf life/expiry date or retest/re-evaluation interval can be carried forward. If primary packaging material or barrier packaging material differs from the original excipient manufacturer's primary packaging material or if the packaged environment varies significantly, then an evaluation of the container and its closure system should demonstrate that it is adequate to protect the excipient from deterioration and contamination during the manufacturer's shelf life/expiry date or retest/re-evaluation interval. Otherwise, a stability assessment is necessary to determine the appropriate shelf life/expiry date or retest/re-evaluation interval for the repackaged excipient. Such assessments should be conducted according to the manufacturer's specifications and test methods.

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3.6 Expiration Dates

Not all excipients have an expiration date, but if one is assigned it should be displayed on the container and should show the period during which the excipient is expected to remain within specifications if stored properly and after which it should not be used. It is established for every batch by adding the shelf life to the date manufacturing began. The expiration date is based on the type of container and storage conditions, so these parameters should be clearly defined. If special storage conditions are needed (e.g., protection from light, oxygen, heat, humidity, etc.), they should be indicated on the labeling because they could influence usability through the expiration date.[5]

The expiration dates for excipients should be established by documented stability tests or long-term stability data. Occasionally, the expiration date may be established by reference to historical data. Stability involves not only the compendial requirements but also changes in performance properties. Excipient stability tests should determine whether possible degradation, changes in molecular weight and distribution, moisture gain or loss, viscosity changes, microbiological contamination, or other possible changes in excipients could occur when the excipient is stored in a specific container–closure type at specific storage conditions. Stability for repackaged excipients can be found under *Section 3.5. Retesting and Shelf Life.*

3.7 Labels, Icons, and Labeling

3.7.1 LABELS AND ICONS

Label-generating systems and processes should be secure, controlled, and documented. Appropriate verification records should be maintained, and each container should be appropriately identified and labeled. Labels applied to individual small containers should be clear, unambiguous, and permanently fixed in the company's established format. The information on the label should be indelible. Alternative methods can be used for bulk containers/transport and should be justified.

The label may include wording or depict icons to highlight storage and transportation handling requirements and hazards (e.g., avoid dropping, maintain specified environmental conditions, etc.). The use of symbols that are recognized by international organizations is recommended (see *Good Storage and Distribution Practices for Drug Products* ⟨1079⟩). During international distribution, the proper language(s) should be used to ensure that handlers understand the requirements set forth on the label.

3.7.2 LABELING

The labeling (which includes both the label and any accompanying documents) should include at least the following information:

- Name of the excipient, including grade and reference to pharmacopeia, as relevant
- If applicable, the International Nonproprietary Name
- Amount (weight or volume)
- Batch number assigned by the original excipient manufacturer or the batch number assigned by the repacker if the material has been repacked and relabeled
- Retest date or expiry date (as applicable)
- Any specified storage conditions, as applicable
- Handling precautions, where necessary
- Identification of the original manufacturing site as agreed with the pharmaceutical customer (see *Section 4.8 Traceability*)
- Name and contact details of the suppliers

SECTION 4: RETURNED GOODS, DISPATCH, TRANSPORT, IMPORTATION, ADULTERATION, AND TRACEABILITY

4.1 Returned Goods

4.1.1 GENERAL

Return of goods by users to suppliers should be reviewed on a case-by-case basis. The distributor should facilitate a root cause analysis and investigation of complaints.

[NOTE—Users should document the reason(s) for return of goods to the supplier.]

Before returning the goods, if the user identifies and confirms unacceptable product quality the user should provide the supplier with the user's supporting documentation, such as tests and investigation results. If requested, the user should also provide product samples used for tests and investigations. The supplier should be provided an opportunity to conduct thorough investigations to confirm the validity of the user's quality complaint. While the investigation proceeds, the user should quarantine the material in accordance with internal standard operating procedures and should store the material in an area specifically designated for returns, with limited access to operations, and well-separated from incoming or released raw materials.

[5] IPEC. *The IPEC Excipient Stability Program Guide 2010.* Arlington, VA: IPEC; 2010. Available at: http://ipecamericas.org/reference-center/document-depot (Accessed June 23, 2017).

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Goods returned by the user because of excess inventory or other causes unrelated to quality can re-enter commerce within the specified shelf life, provided conditions of storage, transportation, and container integrity have been thoroughly reviewed by the supplier and the quality of the excipient has not been compromised in any way. A formal documented review of each returned container and container tamper-evidence device should be done to verify that these match the container configuration when the materials left the supplier's facility.

If the user opens a commercial packaging container for sampling or investigation (related or unrelated to quality issues) and whether any material was taken out or not, each container should be clearly labeled *Opened*. Written documentation should be provided to the supplier confirming that the container(s) were opened and resealed according to GMPs and describing the reasons for opening, amount withdrawn, and how the pack/container was resealed. Documentation of returned goods should contain a detailed description of all such events including repackaging. Returned excipient containers opened by the user should be clearly identified as such and should not be released as pharmaceutical excipients. In exceptional cases, the material can be released as excipient-grade product if a documented thorough investigation shows no risk of product contamination or deterioration. The quality department should release this material.

Users and suppliers should maintain records of all returned goods, including the product name (trade name and chemical name), batch or lot number, reason for the return, quantity returned, and investigation documentation when applicable. In addition, the supplier should record the final disposition of the material. If returned excipients have been held, stored, or shipped under conditions that could compromise product quality (including ingredients, containers, or labeling), the manufacturer should destroy the excipients. Exceptionally, manufacturers can release the excipients if their examination, testing, and investigations prove that the material meets suitable standards of identity, quality, and purity and that GMPs and GDPs have not been compromised.

4.1.2 DISPOSITION OF RETURNED GOODS

The excipient manufacturer's and/or supplier's quality unit should assess the returned product. The options are the following:

- Return to commerce
- Reprocess
- Regrade to a less stringent standard such as technical or industrial grade (non-GMP use)
- Destroy

Only containers that have not been opened should be considered for return to commerce without further action.

If the quality assessment of returned goods leads to their final destruction and if associated batches are potentially implicated, an appropriate investigation should be conducted and documented to show that the quality of the associated batches is not affected.

4.1.3 REPROCESSING

Reprocessing is a manufacturing step, and the requirements of *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩ apply. The requirements of ⟨1078⟩ apply only to those intermediate supply chain entities that undertake reprocessing.

4.2 Shipping and Transportation

4.2.1 SHIPPING

The supplier (the manufacturer or distributor) of pharmaceutical excipients should ensure that the integrity of the pharmaceutical excipient is maintained by the appropriate storage and transport conditions as described in product labeling. After training, staff should follow written procedures for shipping pharmaceutical excipients. These procedures include the requirement to follow the recommended storage and transportation requirements including temperature, humidity, or other special handling precautions.

Actions should be documented when they are performed. Shipping records for pharmaceutical excipients should provide for the following information:

- Date of shipment
- Name and address of the entity that accepted the materials for the transportation
- Mode of transportation
- Name, address, and status of the consignee
- Material name
- Quantity shipped
- Batch number and expiry date
- Required storage and transport conditions (refrigeration, freezing, or controlled room temperature required)
- Shipping code or identification number of the delivery order

When regulatory actions such as FDA Field Alerts or drug product recalls occur, the excipient handler must be prepared to act promptly. Shipping documentation must be sufficient to allow adequate handling of any excipient associated with regulatory action. When reasonable, the shipping schedule for excipients should be documented, and responsibilities can be enumerated in a quality or collaborative agreement between entities to show ownership in the supply chain (Entity A to Entity B; Entity B to Entity C; etc.—see *Section 4.4 Packaging: Tamper-Evident Seals*). The buildings and facilities used to ship materials should be appropriate for their intended use in the storage and handling of excipients (see *Section 3.1 Buildings and Facilities*).

Before loading materials, shippers should inspect the container and vehicle to ensure cleanliness and other consignments (if the shipment is a part load) to ensure no form of contamination is likely to occur. This inspection should be documented

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according to a written procedure. Materials should not be offloaded into other containers or vehicles without the written permission of the material owner or consignee.

4.2.2 TRANSPORTATION

Materials should be transported in a manner that will ensure the maintenance of controlled conditions as specified by the manufacturer. The transport process should not adversely affect the materials or integrity of the packaging. The supplier of transport services must be provided with the required information in order to maintain specified conditions.

The pharmaceutical excipient manufacturer or supplier should agree with the purchaser for arranging transportation. The need for temperature-controlled storage and transport should be determined using a risk-based approach, taking into account the nature of the excipient, results of stability assessments available from the excipient manufacturer or repackager, the supply chain, and the potential risks to the excipient. If temperature-controlled transportation is contracted, the shipper must have a mechanism for noting and reporting temperature excursions. Labeling on containers and transportation documents should detail the environmental conditions in a manner that provides the transporter or receiver with knowledge and immediate identification of these conditions, if required. The responsibility for ensuring that the proper storage conditions are met rests with each entity that handles, stores, or transports the materials.

Pharmaceutical excipients should be stored and transported in such a way that the identity and integrity of the material are retained, the material does not contaminate and is not contaminated by other materials, and adequate precautions are taken against spillage, breakage, misappropriation, and theft. The required storage conditions for pharmaceutical excipients should be maintained within acceptable limits during transportation.

Excipients that are potentially dangerous because of the risk of fire or explosion (e.g., combustible liquids, solids, and pressurized gases) should be stored and transported in safe, dedicated, and secure areas, containers, and vehicles. In addition, applicable international agreements and federal regulations should be followed.

4.3 Tampering or Damaged Materials

Materials that are suspected of being tampered with or damaged must be quarantined immediately, and the manufacturer or distributor should be notified. The disposition of the quarantined material should be determined by the excipient manufacturer or supplier, after consultation with the customer. The excipient may be returned to the manufacturer or supplier. Alternatively, arrangements could be made for local certified destruction of the quarantined material. The supplier should make every effort to prevent these materials from being used until an investigation is completed and the final disposition of the material is determined. Written procedures should guide treatment of excipients that have been tampered with or the identification and handling of damaged material.

4.4 Packaging: Tamper-Evident Seals

A tamper-evident package has one or more indicators or barriers to entry that, if breached or missing, can reasonably be expected to provide visible evidence that tampering has occurred. To reduce the likelihood of successful tampering and to increase the likelihood that any breach will be discovered, the package should be distinctive by design or should employ one or more indicators of or barriers to entry. The term *distinctive by design* means that the packaging cannot be duplicated with commonly available materials or by commonly available processes.

A tamper-evident package may involve an immediate container–closure system in direct contact with the contents (primary packaging), a secondary container–closure system not in direct contact with the contents (secondary packaging), or any combination of systems intended to provide visual evidence of package integrity. For primary packaging in direct contact with the excipient (e.g., paper bags), any leak or break should be considered tampering even if the leak or tear is simply accidental damage. For excipients shipped in bulk, using, e.g., tank cars or containers, other means may be appropriate. However, whatever methods are adopted should provide adequate assurance as to the integrity of the excipient being shipped.

Visual examination of the packaging at each stage in the supply chain should provide evidence of repackaging or tampering with commercial packaging. In addition, the manufacturer's name and address, net weight of the material, material name, batch or packaging number, date of manufacture, and date of retest should be identified on a packaging label. The label should be prominently placed on the package and should be unaffected if the tamper-evident feature of the package is breached or missing.

The tamper-evident feature for excipient packaging should be designed so that it remains intact when handled in a reasonable manner from the time of packaging at the site of manufacture and throughout the supply chain—including but not limited to warehouse storage during various phases of the supply chain, transport, distribution, receipt, and storage at the user's facility until use for drug product manufacture.

The manufacturer should communicate tamper-evident features to the downstream members of the supply chain. If the latter observe any evidence that the tamper-evident feature or other part of the package has been compromised in any way, they should quarantine the material immediately and inform the supplier. Appropriate arrangements should be made with the supplier to return the material promptly with a description of the packaging breach. Alternatively, arrangements can be made by the excipient manufacturer or supplier for the local certified destruction of the quarantined material. The user should ensure adequate protection of the breached packaging during shipment to the supplier and can send photographs of the breached packaging to aid the supplier's investigation.

The supplier is responsible for the integrity of packaging, including but not limited to its tamper-evident features, until ownership of the commercial packages is transferred to the user. Material returned because of breach in packaging should be thoroughly reviewed and investigated by the supplier. The material should not be returned to commerce until the supplier has established that the integrity, identity, quality, purity, and safety of the excipient have not been compromised. The documentation requirement should comply with GMP expectations as well as elements of documentation and investigation suggested in *Section 4.1 Returned Goods*.

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4.5 Where Ownership Begins

The excipient user is responsible for purchased materials throughout the supply chain. The supply chain qualification is documented by audits and COAs for all parties involved in trade and distribution of the materials. Such supply chain qualification and documentation supports the Excipient Pedigree and ownership of the excipient. The pedigree includes documentation of suitable excipient GMPs applied by the excipient manufacturer and suitable GDPs.

Ownership of the materials begins with the original excipient manufacturer and transfers to an intermediary or customer according to agreed-upon terms for insurance costs, transportation, and risk assumption. Such agreements are defined according to International Chamber of Commerce terms (Incoterms).[6] Incoterms are a series of international sales terms that are used to divide transaction costs and responsibilities between buyer and seller and reflect state-of-the-art transportation practices.

4.6 Adulteration

4.6.1 ADULTERATION

Adulteration is defined in the FD&C Act and 21 CFR in Sections 501(a)(2)(B) and 501(b)[7] and 21 CFR 211 for finished pharmaceuticals and Sections 402(a)(3) and (4)[8] and 21 CFR 110 for human food, and in 21 CFR 111 for dietary supplements. These laws and regulations establish the minimum current GMP (cGMP) necessary to prevent adulteration for finished pharmaceuticals, food products, and dietary supplements, respectively. Excipients for pharmaceutical use must be manufactured under appropriate GMPs and must meet the required chemical and physical specifications. In addition to specifications, excipient manufacturers and users have generally agreed quality attributes and limits defined by regulatory agencies, common industry practices, and pharmacopeial expectations. Adulteration or contamination of the products can be monitored and detected by many means including, but not limited to, compliance with these predefined quality expectations.

Adulteration can occur when any possible contamination of a product takes place, e.g., from foreign materials or undesirable microorganisms. The problem of adulteration can be addressed by standard practices supporting cGMPs, such as HACCP, Standard Operating Procedures, and staff training to control product safety and purity. This type of adulteration is the unforeseeable and unintentional type that can be controlled and, at worst, detected before the product leaves the manufacturer's site.

FDA specifies that a product can be considered adulterated when conditions *may* lead to adulteration because it is impossible to test every product for every conceivable contaminant. The safety and purity of substances require that manufacturers should build quality controls into the process rather than relying on QC testing.

4.6.2 INTENTIONAL ADULTERATION

Compared to unintentional adulteration, intentional adulteration is more specific because it requires a willful and knowing violation of regulations and standards designed to protect end user safety. It is the deliberate adulteration of an excipient. When an excipient is deliberately adulterated by substituting a lower-cost material for a material of higher cost, it is considered economically motivated intentional adulteration. Every participant in the supply chain should know and monitor their supply chain for any adulterated materials and take all reasonable precautions to prevent intentional adulteration.[9]

4.7 Importation

Excipients manufactured outside the United States are subject to US FDA and US Customs and Border Protection (CBP) regulations for importation into the United States. The Bioterrorism Act (Public Health Security and Bioterrorism Preparedness and Response Act of 2002, Title III—Protecting Safety and Security of Food and Drug Supply)[10] has further formalized the requirements for importation of foods and drugs into the United States.

Overseas manufacturers of excipients used in drugs, food, and dietary supplements intended for human or veterinary use who intend to export products into the United States are required to follow the FDA, CBP, and Bioterrorism Act regulations. A streamlined process for the importation of excipients used in pharmaceuticals, food, and dietary supplements must be followed and implemented before the imports are allowed into the United States.

The manufacturing facilities and the manufacturers who produce the excipients should be registered with FDA. An FDA registration number is required for importation. In addition, FDA requires information about Prior Notice (PN) of Imports. Upon receipt of the information, FDA grants a PN confirmation number. FDA must confirm PN before the products are shipped, and the PN confirmation number must appear on the customs declaration that accompanies the package. PN information can be submitted electronically to FDA's Prior Notice Systems Interface (PNSI), a free Internet application that allows facilities to provide information regarding the expected imports. PN information also can be submitted via CBP's Automated Commercial System (ACS), a system that processes imports and obtains information needed to make decisions regarding articles entering the United States.

[6] International Chamber of Commerce. Incoterms. http://www.iccwbo.org/incoterms (Accessed June 6, 2011).

[7] FD&C Act. Chapter V: Drugs and Devices. Sec. 501. [21 USC §351] Adulterated Drugs and Devices. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterVDrugsandDevices/ucm108055.htm.

[8] FD&C Act. Chapter IV: Food. Sec. 402. [21 USC §342] Adulterated Food. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterIVFood/ucm107527.htm (Accessed June 6, 2011).

[9] FDA. Public Meeting on Economically Motivated Adulteration. 2009. http://www.fda.gov/NewsEvents/MeetingsConferencesWorkshops/ucm163619.htm (Accessed June 6, 2011).

[10] FDA. *Guidance for Industry: Questions and Answers Regarding the Interim Final Rule on Prior Notice of Imported Food, (Edition 2); Availability.* 2004. http://www.gpo.gov/fdsys/pkg/FR-2004-05-03/pdf/04-10023.pdf (Accessed June 6, 2011).

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CBP processes imports of all goods for entry into United States, including but not limited to pharmaceuticals, food, and dietary supplements. CBP inspects but does not release products regulated by the FD&C Act and delegates the final release responsibility at the port of entry to the FDA for such materials. After reviewing the PN information, FDA may determine that the regulated articles should not be allowed into the United States or may allow conditional import of articles subject to testing and release at the port of entry. In addition, during FDA review at the port the regulated articles must meet all requirements of the FD&C Act and 21 CFR before they are released by FDA to the importer.

Importers of record (individuals or companies) for excipients can contract with a broker to transmit PN information and other documents for them. In this case, the submitter is the person responsible for providing the information, but the broker is the transmitter. Brokers are licensed private individuals or companies regulated by CBP who aid importers and exporters in moving merchandise through CBP. Brokers provide the proper paperwork and payments to CBP for clients and charge a fee for this service. Before brokers apply for a license, they must pass the customs broker examination.

FDA currently uses its Operational and Administrative System for Import Support (OASIS)[11] for making its admissibility determinations to ensure the safety, efficacy, and quality of the foreign-origin products for which FDA has regulatory responsibility under the FD&C Act. OASIS is integrated with CBP's ACS and FDA's PNSI systems to receive information related to imported articles.

US Customs and FDA storage areas at the Port of Arrival may not strictly be in compliance with storage conditions required for certain excipients. Importers of record and brokers who represent importers must ensure that the products are released from Customs and FDA inspection as soon as possible. If release is delayed, FDA generally allows removal by Customs and FDA and quarantine in the importer's warehouse until release. FDA staff who review imports at the port of entry are trained to understand that pharmaceutical excipients must be stored under defined conditions. The manufacturer, the importer on record in the importing country, and brokers have a responsibility in working with Customs and FDA staff to ensure that the storage conditions do not adversely affect product quality during quarantine and review.

4.8 Traceability

4.8.1 TRACEABILITY

The pedigree of the excipient should be tracked from the manufacturer's storage through the final delivery to customers by means of recorded identification. The entire supply chain should provide full traceability (for example, via lot numbers and shipping documents) in order to allow fast and efficient investigation of any quality issue or product recall. Every entity in the supply chain should also take responsibility from the preceding supplier and pass the product to subsequent intermediaries down to the end user. Therefore, the original excipient manufacturer and subsequent handlers should always be traceable, and the information should be available both downstream and upstream in the supply chain. All parties to the excipient supply chain should ensure that the excipient is strictly handled according to GDP at every stage.

To ensure the integrity of the supply chain, intermediaries should use contracts, agreements, inspections, and audits downstream and upstream to monitor compliance with GDP principles. When multiple entities constitute the supply chain for each single batch of excipient, each entity should provide its own supplier's certification documentation (see *Appendix: Definitions and Acronyms*) that represents their manufacture or receipt of the excipient batch through release to the subsequent entity. The total of each entity's supplier certification documentation should represent the entire supply chain from original excipient manufacture through use in the final drug product.

4.8.2 TRACEABILITY-RELATED DOCUMENTS

To ensure traceability, all entities in the supply chain should have clear definitions about the shipping documents to be expected with every delivery. At a minimum the documents for every delivery should provide the following information:

- Name and grade of the excipient
- Lot number(s) assigned by the original excipient manufacturer (see *Section 3.4.2 Repackaging and Labeling Batches*)
- Quality and compliance data (e.g., COA) of the excipient
- Origin of the excipient (manufacturer and manufacturing site)
- Original excipient COA(s) (see *Section 3.4.5 Certificates of Analysis*)
- Entity and site of repackaging (when performed), including opening or relabeling the original excipient manufacturer's packaging for any purpose
- Date of shipment and carrier
- Consignor and consignee

A copy of the COA also should accompany the shipment (see *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩ and *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩).

In the event of repackaging from the original excipient manufacturer's package into another container (including any breach or labeling that does not result in a new package), the identity and address of the repackaging entity should be included in the shipping documents.

Additional data resulting from analyses conducted by entities other than the laboratory of the original excipient manufacturer should be provided, along with a clear indication of the source. Quality documents should facilitate traceability back to the original manufacturer with contact information. The COA issued by the manufacturer should indicate which results were obtained by testing the original material and which results were obtained by other means. A distributor should not change the original title and data of the COA or other quality documents. Whenever possible, the original excipient manufacturer's

[11] FDA. Operational and Administrative System for Import Support (OASIS). 2009.

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documentation should be used, or data transcription should be verified. The original manufacturing site should be identified on the COA.

If any lot mixing is carried out, COAs from manufacturers are no longer valid, and the distributor should perform analyses in its own laboratory or at an approved and qualified contract laboratory. The distributor should supply a COC, and if the blended lot has not been retested, the distributor should inform the customer that the material is a mixture of different original excipient manufacturers' lots, provided that all other repackaging and storage activities are carried out according to GDP.

SECTION 5: EXCIPIENTS USED IN PHARMACY COMPOUNDING

Although analytical testing of incoming components by the compounding pharmacy to confirm quality attributes stated in the COAs is ideal, generally because of resource limitations, compounding pharmacists rely upon the distributors of excipients for assurance of quality and pedigree. Additional guidance on the quality attributes of excipients received by compounding pharmacies can be found in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩. In certain instances *USP–NF* handles compounded preparations differently than commercially manufactured lots. For example, expiration dates are assigned to commercially manufactured products, and beyond-use dates are assigned to compounded preparations (see *Labeling* ⟨7⟩*, Labels and Labeling for Products and Other Categories, Compounded Preparations*). A similar situation is needed for bulk pharmaceutical excipients as not all excipients that are useful in compounding are listed in official compendia (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

State boards of pharmacy regulate pharmacy compounding. USP standards are provided in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, and *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩.

APPENDIX: DEFINITIONS AND ACRONYMS

Acceptance Criteria: The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.

ACS: Automated Commercial System.

Adulterated Material: A material that fails to conform to its purported quality standard or is intentionally contaminated, diluted, or substituted for another substance or which was not manufactured, processed, packaged, distributed and held in conformance with current good manufacturing practice.

Audit: An assessment of a system or process to determine its compliance with the requirements of a particular standard of operation. See also External Audit, Internal Audit, and Third-Party Audit.

Batch (Lot): A defined quantity of processed excipient which can be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production based on time or quantity (e.g., vessel's volume, one day's production, etc.).

Batch Number (Lot Number): A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.

Batch Process: A manufacturing process which produces the excipient from a discrete supply of raw materials processed through discrete unit operations in one mass.

Batch Record: Documentation that provides a history of the manufacture of a batch of excipient.

Blending (Mixing): Intermingling different conforming grades into a homogeneous lot.

Broker: An entity that acts as an intermediary between a buyer and a seller of products or services. Brokers neither buy nor take possession of the products or services.

Calibration: The demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.

CBP: Customs and Border Protection.

CEP (Certificate of Suitability to the *European Pharmacopoeia*): Certification granted to individual manufacturers by the European Directorate for the Quality of Medicines when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

CFR: Code of Federal Regulations.

CFR (Cost and Freight, Named Destination): (Incoterm) Seller must pay the costs and freight to bring the goods to the port of destination. However, risk is transferred to the buyer once the goods have crossed the ship's rail (maritime transport only).

cGMP: Current good manufacturing practices.

CIF (Cost, Insurance, and Freight, Named Destination): (Incoterm) Same as CFR except that the seller must, in addition, procure and pay for insurance for the buyer.

CIP (Carriage and Insurance Paid, Named Destination): (Incoterm) The containerized transport or multimodal equivalent of CIF. Seller pays for carriage and insurance to the named destination, but risk passes when the goods are handed over to the first carrier.

Closed-Container Distributor (Pass-Through Distributor): A distributor who sells only products that are tested, packaged, and sealed in the containers provided by the original manufacturer.

Closed System: A system that is isolated from its surroundings by a boundary so that no material can be transferred across it.

COA (Certificate of Analysis): A document that reports the results of a test of a representative sample drawn from the batch of material that will be delivered.

COC (Certificate of Conformance): A document that certifies that the supplied goods or service meets the required specifications. Also known as Certificate of Conformity and Certificate of Compliance.

Commissioning: The introduction of equipment for use in a controlled manner.

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Compounding: The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice (defined in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Consignee/Consignor: Person or firm (usually the seller) who delivers a consignment to a carrier for transportation to a consignee (usually the buyer) named in the transportation documents.

Contamination: The undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

Continuous Process: A manufacturing process that continually produces the excipient from a continuous supply of raw material.

Contract Giver: A person or organization letting a contract.

Contract Acceptor: A person or organization accepting the terms of a contract and thereby agreeing to carry out the work or provide the services as specified in the contract.

Critical: A process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

Critical to Quality: See *Quality, Critical*.

Cross-Contamination: Contamination of a material or product with another material or product.

Customer: The organization that receives the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

Deviation: Departure from an approved instruction or established standard.

Distributor: An entity that buys products from a manufacturer, takes possession of those products, and resells them to another party or parties. An essential characteristic of a distributor is the order of these transactions. Distributors buy products (i.e., hold inventory) before making sales.

Drug Master File (DMF): Detailed information about the manufacture of an excipient that is submitted to the US FDA.

Drug (Medicinal) Product: The dosage form in the final immediate packaging intended for marketing.

Drug Substance: Any substance or mixture of substances that is intended for use in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

Economically Motivated Adulteration: The fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production for economic gain.

Electronic Signature: A computer data compilation of any symbol or series of symbols, executed, adopted, or authorized by an individual and intended to be the legally binding equivalent of the individual's handwritten signature.

Excipient: Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

Excipient Pedigree: Includes documentation of suitable excipient good manufacturing practices applied by the excipient manufacturer and suitable good distribution practices. See IPEC *Excipient Pedigree White Paper*.

External Audit: (See also *Audit, Internal*, and *Third-Party Audit*.) An audit carried out typically on behalf of an excipient manufacturer's customer by a person or organization that is not the manufacturer or the customer.

Expiry (Expiration) Date: The date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

FCA (Free Carrier, Named Place): The seller hands over the goods, cleared for export, into the custody of the first carrier (named by the buyer) at the named place. This term is suitable for all modes of transport, including carriage by air, rail, road, and containerized/multimodal transport (also called *roll on–roll off*).

FDA: Food and Drug Administration.

FD&C Act: Food, Drug, and Cosmetic Act.

FOB (Free on Board, Named Loading Port): The classic maritime trade term according to which the seller must load the goods on board the ship nominated by the buyer, and cost and risk are divided at ship's rail. The seller must clear the goods for export. The purchaser is then responsible for all further costs associated with transport, importation, and storage until the shipment reaches its destination. The term also is applied to air transport when the seller is not able to export the goods according to the time schedule detailed in the letter of credit. In this case the seller allows a deduction equivalent to the carriage by ship from the air carriage. FOB also can be qualified in other ways. For example, *FOB Factory Gate* means that title and responsibility change as soon as the shipment leaves the supplier's premises.

Forwarding Agents (Freight Forwarders): Agents who assist other organizations or individuals in moving cargo to a destination and are familiar with the import and export rules and regulations of their own and foreign countries, the methods of shipping, and the documents related to foreign trade.

Freight Forwarder: See *Forwarding Agent*.

GDP: Good distribution practices.

GMP: Good manufacturing practices.

Headspace: The volume left at the top of an almost-filled container before sealing.

HACCP (Hazard Analysis Critical Control Point): Hazard Analysis and Critical Control Points has seven principles established by the National Advisory Committee for Microbiological Criteria for Foods to control product safety.

Importer: Either the US owner or consignee at the time of entry into the United States or the US agent or representative of the foreign owner or consignee at the time of entry into the United States who is responsible for ensuring that goods offered for entry into the United States are in compliance with all laws affecting the importation.

Impurity: A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.

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Independent: In the context of internal audits, the quality of being free from any influence, economic or otherwise, from the group, department, or organization under audit.

In-Process Control: Checks performed during production in order to monitor and if necessary to adjust the process to ensure that the material conforms to its specifications. The control of the environment or equipment also can be regarded as a part of in-process control.

In-Process Control/Testing: Checks performed during production to monitor and, if appropriate, to adjust the process to ensure that the intermediate or excipient conforms to its specification.

Intermediate: Material that must undergo further manufacturing steps before it becomes an excipient.

Internal Audit: An audit conducted by an employee of the organization or by an individual from outside the organization, but on behalf of the organization, to determine the effectiveness of a system. (See: *Audit*, *External Audit*, and *Third-Party Audit*).

International Nonproprietary Name: International Nonproprietary Names (INN) facilitate the identification of pharmaceutical substances or active pharmaceutical ingredients. Each INN is a unique name that is globally recognized and is public property. A nonproprietary name also is known as a generic name.

ISO: International Organization for Standardization.

Lot: See *Batch*.

Labeling: The affixing to a container or vessel of a tag or document that contains information about that container and its contents.

Manufacturer/Manufacturing Process: All operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.

Master Production Instruction (Master Production and Control Record): Documentation that describes the manufacture of the excipient from raw material to completion.

Material: A general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.

Nonconforming Material: A material that is deficient in a characteristic, product specification, process parameter, record, or procedure that renders its quality unacceptable, indeterminate, or not according to specified requirements.

OASIS: Operational and Administrative System for Import Support.

Original Excipient Manufacturer: Organization responsible for manufacturing, under appropriate GMPs, the excipient(s) distributed and addressed by this chapter.

Packaging/Repackaging Distributor: A distributor who transfers products from the original packaging or transport vessel(s) provided by the original manufacturer into alternative packaging and sells the products in the alternative packages. See *Distributor* and *Repackager*.

Primary Container–Closure System: The packaging components that come into direct contact with the excipient in the closed, sealed package during storage and transport.

Packaging Material: A material intended to protect an intermediate or excipient during storage and transport.

Packaging: The container and its components that hold the excipient for storage and transport to the customer.

Pass-Through Distributor: See *Closed-Container Distributor*.

PN: Prior notice.

PNSI: Prior Notice Systems Interface.

Primary, Secondary Packaging: See *Packaging/Repackaging Distributor* and *Primary Container—Closure System*. Packaging materials which do not come into contact with the excipient during the normal course of storage and transport of the excipient.

Production: Operations involved in the preparation of an excipient from receipt of raw materials through processing and packaging of the excipient.

QbD (Quality by Design): A systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management. It means designing and developing products and manufacturing processes to ensure a predefined quality.

QMS: Quality management system.

Quality Assurance (QA): The total of the organized arrangements made to ensure that all excipients are of the quality required for their intended use and that quality systems are maintained. See *Quality Unit*.

Quality Control (QC): Checking or testing that specifications are met. See *Quality Unit*.

Quality, Critical: Describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.

Quality Management System (QMS): Management system that directs and controls a pharmaceutical company with regard to quality.

Quality Manual: Describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes. Document specifying the quality management system of an organization.

Quality Unit: See also: *Quality Control* and *Quality Assurance*. A group within a larger organization that is responsible for monitoring and ensuring all aspects of quality. Current industry practice generally divides the responsibilities of the quality control unit (QCU), as defined in the cGMP regulations, between quality control (QC) and quality assurance (QA) functions. QC usually involves (1) assessing the suitability of incoming components, containers, closures, labeling, in-process materials, and the finished products; (2) evaluating the performance of the manufacturing process to ensure adherence to proper specifications and limits; and (3) determining the acceptability of each batch for release. QA primarily involves (1) review and approval of all procedures related to production and maintenance, (2) review of associated records, and (3) auditing and performing/evaluating trend analyses.

Quarantine: The status of materials isolated physically or by other effective means pending a decision about their subsequent approval or rejection.

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Raw Material: A general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.

Recall: See *Retrieval*.

Record: A document stating results achieved or providing evidence of activities performed. The medium can be paper, magnetic, electronic or optical, photographic, another medium, or a combination thereof.

Reevaluation Date (Retest Date, Re-evaluation Interval): The date when the material should be reexamined to ensure that it is still in conformity with the specification.

Recommended Re-evaluation Date: The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. It differs from the Expiration Date because the excipient can be re-evaluated to extend the length of time the material can be used, if supported by the results of the evaluation and appropriate stability data.

Repackager: A person or organization that takes an excipient from the original manufacturer's container and repackages it into different containers. See also *Distributor* and *Packaging/Repackaging Distributor*.

Repackaging: Removal of the excipient from its original container (combination of secondary and/or primary packaging), and transfer to another container.

Reprocessing: Introduction of previously processed material that did not conform to standards or specifications back into the process and repetition of one or more necessary steps that are part of the normal manufacturing process.

Retrieval (Recall): Process for the removal of an excipient from the distribution chain.

Reworking: Subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.

Secondary, Primary Packaging: See *Primary, Secondary Packaging*.

Senior Management: See *Top Management*.

Significant Change: A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

Specification: The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

Stability: Continued conformity of the excipient to its specifications.

Stable Process: A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP and customer specifications) and thus is acceptable for its intended use.

Subcontractor: A person or organization that undertakes work or services on behalf of a different person or organization that in turn is contracted to undertake work or provide services from the original contract giver.

Supplier's Certification Documentation: Specific information and data associated with a single batch of an excipient. Its accuracy is certified by the business entity that has had control of the same single batch of excipient. Supplier's Certification Documentation includes both quality and supply chain data and information. The methods and processes that derive the included data and information should be understood and controlled, and all data and information sources should be traceable. All entities that take possession and responsibility for the excipient batch should provide Supplier's Certification Documentation including the original excipient manufacturer, all distributors, and all repackagers. Special attention and clarity should be applied within the Supplier's Certification Documentation in any event that breaches the original manufacturer's packaging and/or labels (including addition of new labels).

Third-Party Audit: An audit conducted by an individual from outside the organization and who is neither a supplier nor customer of the organization, e.g., a certification body, to determine the effectiveness of a system.

Top Management: Person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and depends on how the quality management system is organized.

Traceability: Ability to determine the history, application, or location that is under consideration, e.g., origin of materials and parts, processing history, or distribution of the product after delivery.

Trader: An entity that buys products from a manufacturer, may or may not take possession of the products, and resells them to another party or parties. [NOTE—In the case of traders, the sale usually is made before product purchase.]

User: A person or organization that uses pharmaceutical excipients to manufacture pharmaceutical intermediates or finished products.

Validation: A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

⟨1207⟩ PACKAGE INTEGRITY EVALUATION—STERILE PRODUCTS

1. INTRODUCTION

This chapter provides guidance on the integrity assurance of nonporous packages intended for sterile pharmaceutical products. Background instruction is provided on the topics of leaks, leakage rate, and package sealing/closure mechanisms. Explanation is given as to how packages that conform to specified leakage limits help to ensure the contained product meets and maintains sterility and relevant physicochemical specifications. The integration of package integrity assurance as a key component of the entire product life cycle is stressed. Guidance in the selection, validation, and use of leak test methodologies as well as package seal quality tests is included. Detailed recommendations are presented in three subchapters listed below:

- *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩
- *Package Integrity Leak Test Technologies* ⟨1207.2⟩
- *Package Seal Quality Test Technologies* ⟨1207.3⟩

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At the end of this chapter, the *Glossary* section defines terms as they are used in the context of this set of four general information chapters.

The term product–package refers to the container–closure system plus the product contents. The container–closure system consists of the primary packaging components, those components that are or may be in intimate contact with the product, as well as secondary packaging components vital to ensuring correct package assembly, for example, the aluminum cap used to seal a stoppered vial package.

Suitable container–closure systems adequately store and protect the contained pharmaceutical product. Thus, sterile product–package integrity is the ability of a sterile product container–closure system to keep product contents in, while keeping detrimental environmental contaminants out. Specifically, leaks of concern for sterile product–packages include the following three categories described in *Table 1*. In other words, the leaks of concern for a given product–package are a function of the degree of package protection demanded by the product to ensure that all relevant product physicochemical and microbiological quality attributes are met through product expiry and use.

Table 1. Product Quality Risks Posed by Leaks of Concern

Leaks of Concern	Product Quality Risks Posed by Leaks
Capable of allowing entry of microorganisms	Failure of product sterility quality attribute
Capable of allowing escape of the product dosage form or allowing entry of external liquid or solid matter	Failure of relevant product physicochemical quality attributes
Capable of allowing change in gas headspace content. For example, loss of headspace inert gases (e.g., nitrogen), loss of headspace vacuum, and/or entry of gases (e.g., oxygen, water vapor, air).	Failure of relevant product physicochemical quality attributes and/or hindrance of product access by the end-user

Package integrity is synonymous with container–closure integrity; these terms are used interchangeably throughout this chapter. In the past, to say a sterile product–package has container–closure integrity commonly meant that the package either had passed or was capable of passing a microbiological challenge test. This guidance chapter defines the concept of container–closure integrity more broadly, encompassing the absence of all package leaks that risk product quality. By this definition, a package is considered to have integrity if it allows no leakage greater than the product–package maximum allowable leakage limit. In other words, the largest and smallest leaks of concern are absent.

Leakage differs from permeation. Leakage is the unintentional entry or escape of matter (solids, liquids, or gases) through a breach in a package wall or through a gap between package components. Leakage can also refer to the leaking matter itself. Leakage flow rate is a function of the absolute and/or partial pressure gradient of leaking matter that exists across the package barrier. In the context of this chapter, permeation is the passage of fluid (e.g., gas) into, through, and out of a nonporous package wall. Permeation, not leakage, occurs when only a small fraction of molecules is able to move through a barrier by way of any one hole. A nonporous package is able to permit permeation, but not the volumetric flow of air. Package permeation test methodologies fall outside of the chapter scope and are not described.

Package integrity tests are leak test methods. A leak test is a method that detects the presence of (and in some cases, the magnitude or location of) a package breach or gap.

Package seal quality tests are techniques used to characterize and monitor the quality and consistency of a package seal or closure system parameter, which can influence the package's ability to maintain integrity. An example is the peel force test widely used to test seal strength. Seal quality tests are not leak tests but can play a valuable role by monitoring a characteristic of the seal itself, the package materials, the package components, and/or the processes required to create the seal or closure mechanism.

Integrity test methods vary not only in their application and detection limit, but also in terms of detection range, precision, and specificity, for example. No one test is appropriate for all packages or for all leak testing applications. Selection criteria for package integrity test methods, as well as method comparison aids, are presented to guide the user in the selection process.

Leak tests, even many commonly recognized industry standard testing approaches, require optimization and validation for each product–package application. A science- and risk-based approach may allow some tests to be leveraged for broader application under certain circumstances. For example, small differences in product formulation or package design and materials may permit the use of one test for multiple product–packages. Package integrity verification plays an important role throughout the product life cycle, starting with product development and continuing through marketed product stability studies.

2. SCOPE

The package integrity leak tests and seal quality tests outlined in this chapter apply to the testing of both large and small volume container–closure systems for sterile pharmaceutical dosage forms. Such package systems include, but are not limited to, the following examples.

- Vials or bottles closed with elastomeric closures or screw-thread caps
- Form-fill-seal plastic or glass ampules
- Syringes or cartridges
- Flexible bags or pouches
- Packages for some drug/device combination products (e.g., packages cased inside autoinjectors)

Outside the chapter scope are packaging systems and processing equipment used in the preparation, storage, and manufacture of sterile pharmaceutical products. Examples include containers for active pharmaceutical ingredients and containers for product intermediate or final bulk product.

Outside the chapter scope are packages used for sterile diagnostic products or medical devices, and some packages used for sterile drug/device combination products.

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Outside the chapter scope are products having a primary package composed of porous barrier package materials, i.e., materials designed with pores or openings to allow volumetric air flow while preventing airborne microbial contamination.

3. LEAKS AND LEAKAGE RATE

Leaks are commonly conceptualized as holes of a defined diameter, or channels of distinctive diameter and length, although leaks that occur naturally are generally complex, multicavity tortuous paths and are rarely uniform in size or shape. Even artificially created leaks such as laser-drilled defects (also called holes) used for leak-test method development and validation are irregular in size, shape, and depth. When stating the size of a leak, it is important to define the measurement approach. In some cases leaks are measured dimensionally, but quite often, leak size is determined based on gaseous leakage rate. For example, a package wall laser-drilled defect having a nominal diameter of 5 ± 2 µm may have been size-certified by matching the airflow rate through the drilled defect to that of a 5 ± 2 µm hole present in a thin metal plate reference standard when pressurized with dry air at specified differential pressure and temperature conditions.

Gaseous leakage rate is a measure of the rate of gas flow (in mass or volume units) that passes through a leak path under specific conditions of temperature and the concentration or pressure differential across the barrier wall. Therefore, gaseous leakage rate has dimensions of pressure multiplied by volume, divided by time. The international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$). These leakage measurement units refer to the quantity of leaking gas ($Pa \cdot m^3$) per unit of time. When a leakage rate is described and no test conditions are noted, standard conditions of one standard atmosphere differential pressure with dry air at 25° are assumed. For a more complete discussion of gaseous leakage rates and units of measure refer to reference *1*.

Unintentional leaks in packages are often detected or sized using gas as a tracer element. Given a situation in which a tracer gas partial pressure difference exists across the package barrier wall and no absolute pressure difference exists, gaseous leakage is predominantly diffusional in nature as the tracer gas moves from a region of higher concentration to a region of lower concentration. For example, the headspace in a vial package low in oxygen concentration and at ambient pressure at time of closure will exhibit a rise in headspace oxygen concentration over time as a function of diffusion rate (relative to package leak size) plus permeation rate (relative to permeability through the package). Such a change can be monitored instrumentally, as is discussed later in the chapter.

Given the situation in which an absolute pressure difference exists across the package barrier wall, gas leakage through package gaps is more rapid (flux being primarily convective) as gas moves from the higher pressure region to one of lower pressure. For most package materials, the permeation rate of the gas of concern through the package wall is insignificant in comparison. In this example, the rate of leakage into the package is determined by monitoring the change in headspace absolute pressure as a function of time.

For many instrumental leak test methods described in this chapter, gas flow into or out of package leaks is induced by exerting a pressure gradient across the package. In this way, a wide range of leak types and sizes can be identified.

Liquid leakage rate is a measure of the volume of liquid that moves through a leak path as a function of time under specified conditions of temperature and absolute differential pressure across the barrier wall. Liquid movement through a leak path occurs only when leak size/shape, package materials of construction, the absence of leak obstruction, tracer liquid composition, and test parameters all work together. All sterile product–packages within the chapter scope are intended to prevent liquid leakage and block microbial entry, thus it is the absence of liquid leakage flow or the absence of liquid in the leak path, rather than the rate at which liquid leakage occurs that is typically verified in relevant package leak tests. Liquid leakage rate measurements are only useful in measuring larger size leaks.

4. CLOSURE TYPE AND MECHANICS

An understanding of closure mechanics makes it possible to better characterize, monitor, and test packages for integrity. This section discusses various closure systems and how each type functions to ensure package integrity.

4.1 Physically Mated Closures

Closure can be achieved by the close physical mating of two surfaces that often are dissimilar in material composition. Examples include the interference fit of a plunger inserted in a syringe barrel, the compression fit of an elastomeric closure capped onto a vial finish surface, and the application force of a screw-cap torqued onto a bottle. Physically mated surfaces are not bonded together; therefore, a tiny gap exists even between well-closed components. Nevertheless, when properly designed and assembled, closure systems fit together such that liquid leakage (and microbial ingress) is prevented and gas migration is limited. Regarding screw-thread closures such as those used for ophthalmic-product dropper bottles, the barrier to leakage is afforded by inner cap surfaces pressing against the package opening, in some cases aided by a secondary gasket or plug. The winding path afforded by the threads of a screw-cap does not provide an optimal barrier to gas or liquid leakage, or to microbial ingress in the event of liquid presence in the cap threads.

4.2 Physicochemically Bonded Closures

Seals are formed when two similar or dissimilar surfaces are physicochemically bonded together. One example is the formation and sealing of contiguous containers from a single material (e.g., glass or plastic ampules). In other form, fill, and seal systems, sheets of material are mated by means of a heat or ultrasonic welding process. Two dissimilar materials may be joined using an intermediate bonding material. Fully fused seals inherently block liquid leakage and microbial ingress; however, gaseous leakage and permeation may occur.

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4.3 Multiple-Dose Package Closures

Multiple-dose package closures are designed to allow product access while limiting microbial ingress and product leakage between doses. For example, elastomeric closures for parenteral products requiring needle-puncture access are designed to offer reseal protection against microbial ingress and product leakage. Some multiple-dose ophthalmic dosage form package closures are designed with filters, plugs, or other mechanical means that allow the product to be dispensed while restricting microbial ingress and product leakage. For example, ophthalmic product–package closure systems have been designed to automatically pinch shut between intermittent dosing to limit microbial entry and product loss.

5. PRODUCT–PACKAGE QUALITY REQUIREMENTS AND THE MAXIMUM ALLOWABLE LEAKAGE LIMIT

As noted in *Introduction*, package integrity is necessary to maintain product critical quality attributes within physicochemical label-claim specifications and to ensure product sterility until time of use. Detrimental contaminants include microorganisms and any substances that threaten patient safety or product quality. Product leakage can cause a product to fail content or potency specification limits. For certain products to maintain product physicochemical stability, the package needs to maintain a headspace of nonreactive gases and/or low water vapor content, sealed under atmospheric or reduced-pressure conditions. Headspace vacuum conditions may also be necessary to facilitate product ease of use, e.g., product reconstitution using a diluent injected into the container.

Most package types display very low but definite gaseous leakage flow through the gap that exists even between well-fitted closures. Therefore, it is not practical to require that packages be absolutely leak-free. Rather, it is the significance of leakage in relation to product quality that needs to be considered. In other words, the package should not permit leakage beyond the product's maximum allowable leakage limit. Such leaks of concern should be absent.

Identifying the maximum allowable leakage limit for a product–package is a science- and risk-based decision. The smaller the leak path, the less likely the product can escape and the less likely microorganisms or other contaminants can enter. Eventually, leak paths may be so small that only headspace gas exchange is realistically possible. Package construction and assembly, package contents, and the range of environments a given product–package may be exposed to during its life cycle are to be considered when specifying the maximum allowable leakage limit.

There are two major product–package quality requirement categories used when specifying the maximum allowable leakage limit and one subcategory that applies only to multiple-dose packages. These are discussed below.

5.1 Sterility and Product Formulation Content must be Preserved; Gas Headspace Content Preservation is not Required

This category includes product–packages for which the maximum allowable leakage limit correlates to the prevention of product formulation escape, or product contamination by external liquid or solid matter or by microorganisms, while gas headspace preservation (i.e., ambient pressure air) is of no concern. The smallest leak paths that allow only limited gas exchange are irrelevant as they pose no real risk to product quality.

Considerable published research exists exploring rigid package leaks and their relationship to risk of aqueous solution leakage and/or microbial ingress. A key study series found orifice leaks of approximately 0.1 µm in nominal diameter (using micropipettes) placed in rigid packages demonstrate a small risk of aqueous liquid passage, while orifice leaks as small as approximately 0.3 µm in nominal diameter first demonstrate some risk of microbial ingress by liquid challenge (*2,3*). For a summary of similar studies comparing risks of microbial ingress and liquid leakage to leak size and type, the reader may refer to reference *4*.

Given this body of evidence, a maximum allowable leakage limit of less than 6×10^{-6} mbar · L/s (measured by helium mass spectrometry in the vacuum mode) can be adopted for products in this category packaged in rigid container–closure systems. This leakage rate equates to the presence of an orifice of nominal diameter of between 0.1 and 0.3 µm. At this leakage rate, the probability of microbial ingress was determined to be <0.10 (*2*). Selecting this conservative maximum allowable leakage limit will ensure a low risk of microbial ingress and liquid leakage and can eliminate the need to perform additional microbial ingress or liquid challenge studies as a function of leak size.

For other container systems such as those made using flexible materials or those with complex, lengthy closure/seal interfaces, or those meant to contain a product of markedly greater leakage potential, the risk of microbial ingress or liquid passage through leak paths is not as widely publicized or perhaps understood. In such cases, where the relationship between defect size/type to the risks of microbial ingress and/or liquid passage is less prescriptive, a study exploring these relationships could be useful. Test results can be used to establish a meaningful maximum allowable leakage limit for the given product–package system, which can be employed for package integrity verification by other validated leak test methods of choice. Once established, this maximum allowable leakage limit can be applied to similar product–packages with appropriate justification.

Finally, before classifying a product–package in this category, one may consider the potential impact of product life cycle processing, storage, distribution and use scenarios on package integrity. For example, elastomeric closures have been found to shrink and lose their viscoelastic properties during ultra-cold storage (≤–80°) to such an extent that gas influx into stoppered vial packages may occur. During warming, package closure is restored, trapping gases and notably raising internal package pressure. In such cases, gas headspace preservation may be a product quality concern, even if the product does not require specific headspace content or pressure for optimum stability.

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5.2 Sterility, Product Formulation Content, and Gas Headspace Content must be Preserved

The second category includes product–packages for which the package should prevent product formulation escape and product contamination by external liquid or solid matter or by microorganisms, but in addition, the package must preserve the gas headspace content or absolute pressure. Preservation of headspace content and/or pressure is needed to maintain product stability within physicochemical specification limits, and/or to aid end-user product access. Thus, the maximum allowable leakage limit for such products is likely more stringent than that described in the first category. Although outside the chapter scope, the influence of gas permeation through the package itself may also need to be considered when establishing the maximum allowable leakage limit as permeation plus leakage can impact package headspace content.

The maximum allowable leakage limit for products in this category may be expressed in terms of the maximum allowable package headspace content or pressure change as a function of time. This limit, established for each product–package system, may be applied to other similar product–packages systems with appropriate justification.

5.3 Sterility must be Preserved; Product Access is Required

This subcategory represents a quality requirement applicable only to multiple-dose product–packages included in either of the above two categories. Once the product–package has reached the end-user and the closure has been activated or otherwise compromised to allow dosage access and delivery, the maximum allowable leakage limit at this life cycle phase (called the in-use maximum allowable leakage limit) is defined in terms of microbial ingress and product loss prevention between and during dosage access. For example, elastomeric closures of multiple-dose vials or cartridges containing antimicrobial-preserved parenteral products are designed to afford reseal protection against microbial ingress and product formulation leakage as product is accessed via needle puncture as well as between doses.

To establish the in-use maximum allowable leakage limit for multiple-dose package closure systems, a study will likely be required to explore the relationship between product access attempts, product loss risk and/or microbial ingress risk, versus leakage measurement by an alternative adequately sensitive leak test method of choice. These data can be used to establish a meaningful in-use maximum allowable leakage limit expressed in units of measure reflective of the preferred leak test method and that will assure that product loss and microbial ingress risk during use is minimal. The in-use maximum allowable leakage limit established for a given product–package may be applied to other similar product–packages systems with appropriate justification.

6. INHERENT PACKAGE INTEGRITY AND THE PACKAGE INTEGRITY PROFILE

Inherent package integrity is the leakage characteristic of a well-assembled container–closure system using no-defect package components. Inherent package integrity is first determined during product–package development and qualification and is a measure of the leak tightness of a container–closure system, given anticipated variables of material composition, dimension, processing, and assembly. Inherent package integrity may also be determined as a function of anticipated final product storage, distribution, and use. Acceptable inherent package integrity for a container–closure system conforms to the specific product–package maximum allowable leakage limit. Inherent package integrity is expressed in terms that allow a meaningful comparison to the maximum allowable leakage limit.

Confirmation that the inherent package integrity conforms to the maximum allowable leakage limit is the first step in product–package integrity verification. Verification of package integrity continues throughout the product life cycle. The package integrity profile is an ongoing database of product life cycle package leak and seal quality test results. This profile provides information regarding package integrity given operative variations in package component design and material; package assembly and processing; and product storage, distribution, and stability. These concepts are more fully explored in *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩, *Test Instrument Qualification, Method Development, and Method Validation*.

In summary, a product–package system having integrity is one in which the inherent package integrity conforms to the required product–package maximum allowable leakage limit. Further, the package integrity profile database operates as a risk management tool to ensure that finished product container–closure systems are intact, able to block microbial ingress, restrict loss of product contents including critical headspace gases, and prevent entry of detrimental gases or other substances, thus ensuring that the product meets all relevant physicochemical and microbiological label-claim specifications through expiry and final end-use.

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GLOSSARY

For definitions of container, materials of construction, packaging component, packaging system, primary packaging component, and secondary packaging component, see *Packaging and Storage Requirements* ⟨659⟩. In the context of this chapter and its subchapters, the following definitions relevant to packaging and package integrity methods, seal and closure mechanisms apply. For definitions of specific leak test and seal quality test methods, refer to ⟨1207.2⟩ and ⟨1207.3⟩, respectively.

Accuracy: The accuracy of a leak test method is a measure of the ability of the method to correctly differentiate packages that leak above the claimed detection limit from those that leak below this limit (do not leak); a measure of false positive and negative occurrence. Alternatively, for those methods that deliver an outcome that is a direct quantitative measure of gas leakage rate (or of gas content or pressure), accuracy is a measure of the method's ability to produce an outcome comparable to a true standard, such as a nationally recognized gas standard.

Container–closure integrity: Container–closure integrity is the ability of a package to prevent product loss, to block microorganism ingress, and to limit entry of detrimental gases or other substances, thus ensuring that the product meets all necessary safety and quality standards. Synonymous with *Package integrity*.

Container–closure integrity test: A container–closure integrity test is any package leak test (either physicochemical or microbiological) that detects the presence of a package breach or gap. Some tests may also be able to identify the leak magnitude and/or location. The term container–closure integrity test is synonymous with package leak test or package integrity test.

Container–closure system: See *Packaging and Storage Requirements* ⟨659⟩, *General Definitions, Packaging System*.

Detection limit: The leak test detection limit is a measure of test method sensitivity. The detection limit is the smallest leakage rate (or leak size) that a leak test method can reliably detect. Also called limit of detection.

Deterministic leak test method: A deterministic leak test method is one in which the leakage event being detected or measured is based on phenomena that follow a predictable chain of events. In addition, the measure of leak detection is based on physicochemical technologies that are readily controlled and monitored, yielding objective quantitative data.

In-use maximum allowable leakage limit: See *Maximum allowable leaking limit*.

Inherent package integrity: Inherent package integrity is the leakage rate (or leak size) of a well-assembled container–closure system using no-defect package components. Inherent package integrity is a measure of the leak tightness of a container–closure system, given anticipated variables of material composition, dimension, processing, assembly; package storage, distribution and use.

Leak: A leak is a breach in a package wall or a gap between package components that is capable of permitting the passage of gas, liquid, or solid matter. Leak is synonymous with leak path.

Leakage: Leakage is the unintentional escape or entry of matter (solid, liquid, or gas) through a breach in the package wall or through a gap between package components. Leakage can also refer to the substance that enters or escapes from a compromised package. For example, "Leakage from the cracked container stained the package label."

Leakage rate: Gaseous leakage rate is a measure of the rate of gas flow (in mass or volume units) that passes through a leak path under specific conditions of temperature and absolute pressure or concentration differential. Leakage rate has dimensions of pressure multiplied by volume, divided by time. For example, the international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$). Other common units of measure include standard cubic centimeters per second ($std \cdot cm^3 \cdot s^{-1}$ or sccs) and mbar liters per second ($mbar \cdot L \cdot s^{-1}$). Liquid leakage rate is a measure of the volume of liquid that moves through a leak path as a function of time under specified conditions of temperature and absolute differential pressure across the barrier wall. Liquid leakage rate measurement, possible for larger leak tests, requires that leak size, materials of construction; absence of leak obstruction; tracer liquid composition; and test parameters work together to ensure liquid movement through the leak path.

Linearity: Leak test method linearity is the ability of the method to elicit test results that are mathematically proportional to leak path size or leakage rate.

Master: A master is a package prototype, model, or facsimile made to simulate an actual package in shape and design. Masters may be made of solid plastic or metal, or they may be simply a designated container–closure unit. Masters are designed to simulate a no-leaking package, and are often used in leak test system suitability tests to verify instrument performance.

Maximum allowable leakage limit: The maximum allowable leakage limit is the greatest leakage rate (or leak size) tolerable for a given product–package that poses no risk to product safety and no or inconsequential impact on product quality. The maximum allowable leakage limit for a sterile pharmaceutical dosage form package will ensure the content's sterility, preserve product contents, and prevent entry by detrimental gases or other substances, thus ensuring that the product meets relevant physicochemical and microbiological specifications through expiry and use. For multiple-dose product–packages, the in-use maximum allowable leakage limit is defined as the degree of protection demanded of the closure to limit microbial ingress and product formulation leakage between and during dosage access.

Microbiological challenge test: A microbiological challenge test is a package leak test whereby package integrity is evaluated by exposing containers filled with growth-supportive media to microorganisms suspended in submersion media (a liquid-borne challenge test). Leakage is evidenced by the subsequent growth of the challenge microorganisms in the package contents. Synonymous with microbial challenge test.

Negative control: A negative control is a package with no known leak. Negative controls used for leak test method development and validation studies represent packages optimally assembled using normally processed components. Negative controls should duplicate the container–closure system of the product under integrity investigation. For some methods, it may be necessary for negative controls to simulate test product headspace and formulation content as well.

Nonporous: Nonporous packaging does not have pores or openings to allow volumetric air flow. In other words, NMT a small fraction of molecules is able to pass through any one hole. Nonporous materials may be impermeable, semi-permeable, or permeable to the passage of fluid through the package barrier wall.

Nominal diameter: In the context of this chapter, nominal diameter is a means of expressing the size of a package leak. Package leaks are typically irregular tortuous paths or matrices that cross a package wall or barrier. Such leaks are difficult to size in accurate or meaningful terms. The nominal diameter of a leak path is defined as the width of an orifice of relatively short

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depth, through which gas (e.g., dry air) at equivalent airflow rate may pass when subject to one atmosphere differential pressure at controlled temperature conditions.

Package integrity: See *Container–closure integrity*.

Package integrity test: See *Container–closure integrity test*.

Package integrity profile: The package integrity profile is a database of product life cycle package leak and seal quality test results that denotes product–package integrity given operative variations in package component design and material; package assembly and processing; and product storage, distribution, and stability.

Package leak test: See *Container–closure integrity test*.

Package seal quality: Package seal quality relates to the consistency of a package seal's performance within required specification limits. Examples of package seal quality attributes include heat seal bond strength and capped vial package residual seal force.

Package seal quality test: A package seal quality test is used to characterize and monitor the quality of a product–package seal or closure system to ensure that package assembly is consistently kept within established limits. Package seal quality tests are not and cannot substitute for leak tests. However, they can provide some assurance of the package's ability to maintain integrity by monitoring a characteristic of the seal itself, the package materials, the package components, and/or the processes required to create the seal or closure mechanism. Examples include the seal peel force test and the capped vial package residual seal force test.

Permeation: Permeation is the passage of fluid into, through, and out of a nonporous package wall. Permeation, not leakage, occurs when the package barrier has no holes large enough for more than a small fraction of molecules to pass through any one hole.

Physicochemical package integrity test: A physicochemical package integrity test is a leak test that detects the presence of a package leak, or detects/measures package leakage rate, via physical or chemical means. All leak test methods that do not use microorganisms for leak detection are physicochemical leak test methods.

Porous: A porous package or package material has pores or openings to allow volumetric air flow while still blocking airborne microbial passage. Examples include coated paper or spun-bonded polyolefin barrier materials that permit package penetration by ethylene oxide gas or steam.

Positive control: A positive control is a package with a known, intentional defect. Positive controls used for leak test method development and validation studies should duplicate study negative controls in terms of materials of construction, package assembly, and component processing. Positive controls are used during leak test method development and validation. Some methods require the use of positive controls during routine testing as well.

Precision: Leak test method precision is a measure of the method's ability to yield reliable, repeatable data. Precision includes repeatability (repeat testing of a single homogeneous test sample population set), ruggedness (within laboratory tests performed by multiple operators on multiple days, using multiple instruments; also known as intermediate precision), and reproducibility (tests among multiple laboratories).

Probabilistic leak test method: A probabilistic leak test method is the converse of a deterministic leak test method, being stochastic in nature. Probabilistic tests rely on a series of sequential and/or simultaneous events, each associated with random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate large sample sizes and rigorous test-condition controls to obtain meaningful results. Typically, sample size and test condition rigor are inversely related to leak size.

Product: The pharmaceutical product includes the pharmaceutical formulation as well as the packaged headspace, which may consist of ambient air or nonreactive gases with specified water-vapor content under full or sub-atmospheric pressure conditions.

Product–package: The product–package includes the primary package with critical secondary components (the container–closure system) plus the packaged product (the contents).

Qualitative measure of analysis: A qualitative measure of analysis for leak testing is a measurement approach based on a subjective evaluation of some quality, attribute, or characteristic of the test sample. Visual inspection is an example of a qualitative measure of analysis.

Quantitation limit: The quantitation limit is a leak test method characteristic defining the lowest leakage rate or leak size that can be differentiated with accuracy and precision under the stated experimental conditions.

Quantitative measure of analysis: A quantitative measure of analysis for leak testing is a measurement approach based on objective, numeric data that either directly or indirectly correlates with leak presence, leak location, or leakage rate. Examples include the mass-of-gas-per-time reading generated by the helium mass spectrometry tracer-gas leak test, or the pressure reading as a function of test time measurement produced by the vacuum-decay method.

Range: The range of a leak test method is that interval between the smallest to largest leak size (or leakage rate) that can be detected by a given leak test method with a suitable level of accuracy and precision.

Robustness: Robustness of a leak test method is the method's ability to accurately identify leaking versus nonleaking packages despite small but deliberate variations in procedural parameters, providing an indication of the method's suitability during normal usage.

Specificity: The specificity of a leak test method is the ability of the method to accurately differentiate leaking and nonleaking packages, despite interfering factors that may cause false detection.

System suitability: System suitability is a manner of ensuring that the leak test method including all factors, which may be subject to variability, that may impact test results (such as instrumentation, analysts, test sample preparation steps, and the test environment) are adequately controlled and maintained in such a fashion that the method is rugged and robust.

System suitability test: A system suitability test is a test to verify that the leak test method and all key factors that may impact test results are correctly controlled and set prior to method performance.

Tortuous path: As applied to leaks, a tortuous path is a convoluted, complex leakage pathway. Most naturally occurring leaks, such as cracks and tears, are tortuous in nature, rather than pristine holes. As applied to sealing mechanisms, a sealing material that has tortuous barrier qualities can block microbial entry. [NOTE—The winding path afforded by the threads of a

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screw-cap (e.g., an ophthalmic dropper bottle closure) does not provide an optimal barrier to gas or liquid leakage, nor does it provide an optimal barrier to microbial ingress in the event of a liquid presence in the cap threads.]

Type defect: A type defect is a positive-control package that represents realistic package flaws. Type-defect positive controls may be included in leak test method feasibility and development studies before method validation. An example of a type defect is a heat seal wrinkle or a loose cap. Type defects are inherently irregular in size and shape and are often described qualitatively instead of being described in terms of leak size or leakage rate.

⟨1207.1⟩ PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE —TEST METHOD SELECTION AND VALIDATION

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1. INTRODUCTION

Package Integrity Evaluation—Sterile Products ⟨1207⟩ provides guidance in the integrity assurance of product packages. This chapter describes package integrity verification during three product life cycle phases: 1) package development, and package processing and assembly validation; 2) product manufacturing; and 3) commercial product shelf-life stability assessments. Further, this chapter provides information on how to select, develop, and validate leak test methods.

2. PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE

Appropriate packaging for a sterile product can be determined on the basis of a knowledge-gaining effort conducted during the product's life cycle.

2.1 Development and Validation

2.1.1 PACKAGE DEVELOPMENT

Package development begins with the preparation of a product–package profile (e.g., user requirements specification), which considers the product end use, stability requirements, and method of manufacture, as well as the anticipated storage, shipment, and distribution environments. This profile also defines the proper product–package quality requirements and the package's maximum allowable leakage limit (see *Package Integrity Evaluation Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit*). With this prospectively developed body of information, one can select each package component's materials of construction, choose suitable component sources, and establish critical physical attributes and component dimensional tolerances. Each component material, with its critical dimensional tolerances, directly affects the integrity of the final packaged product. Assurance of package integrity originates from the use of appropriate materials, accurate

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and optimum closing properties, matching dimensional fit, appropriate multi-component stack heights and tolerances, and consistent control of processes used to assemble the closed package.

The manner in which the package is processed, formed, or assembled is an important consideration in package integrity assurance. A preliminary assessment of package integrity at the end of the development phase under conditions representative of the marketed product manufacturing system is prudent.

These conditions include processes such as sealing operations and component sterilization. Where possible, processes are performed according to established and approved user requirement specifications. Establishing appropriate physical characteristic specifications for container materials, considering lot-to-lot variations, can ensure that the most extreme processing conditions anticipated (e.g., multiple sterilization cycles) do not physically damage materials in a manner that would adversely affect package integrity.

Finally, the robustness of the manufactured product–package system may be evaluated during the development phase by exposing a representative number of product samples to specified storage, shipment, distribution, and final product-use environments. These efforts may include studies that evaluate package integrity at the extremes of the finished product–package profile, not simply at optimal conditions. Given the complexity of some sterile packages, it may be useful for package integrity development studies to incorporate multi-point analyses of test packages manufactured within specified process parameters. Utilizing a common container–closure for which the producer has significant experience and process knowledge can supplement the overall development process and may help reduce the efforts needed. A battery of integrity and seal quality test methods may be employed during product–package development, starting with techniques able to measure the product's inherent package integrity. Inherent package integrity of a viable package system conforms to the product's maximum allowable leakage limit.

Test package quantities for inherent package integrity verification may vary on the basis of: 1) the complexity of the product–package, 2) the specifics of the user specification requirements, and 3) the prior experience of the producer. Test quantity choice is also influenced by the confidence that can be placed on the package integrity test results as well as the level of integrity assurance desired. In some cases inherent package integrity verification may be more readily and economically determined by using empty or placebo-filled container–closure systems, thereby enabling larger sample quantities to be tested by the most sensitive and quantitative leak test methods without the risk of product formulation interference with the test method.

The outputs of the packaging development phase include the final user requirement specifications, which form the basis of production purchasing specifications for package components. Also during the development phase, the final equipment user requirement specifications are developed for package material cleaning, sterilization, and forming; sealing or assembly equipment; and allied materials supply and component feed systems. These user requirement specifications provide purchase specifications for the acquisition of equipment or for the vetting of potential contract manufacturers.

2.1.2 PACKAGE PROCESSING AND ASSEMBLY VALIDATION

Final confirmation of acceptable inherent package integrity is generally part of a larger process validation activity for the overall production process. The scope depends upon the product type and whether the organization has previous experience with the container–closure system.

All processes germane to the sterilization and formation of a package having integrity are to be evaluated against the user requirement specifications established in the package development phase, including likely process extremes. For example, inherent package integrity verification may consider extremes of package assembly variables such as line speed, heat-sealing temperature, screw-cap application torque, and vial-capping forces, as well as resterilization processes, labeling, and secondary and tertiary packaging processes. Validation test requirements and scope should fit the statistical requirements and capabilities of each process, taking into account both package and package-line complexity, as well as prior experience with similar product–packages.

Testing done during technical transfer from the product development site to the manufacturing sites will assist in determining whether the user requirement specification targets and the control ranges established in development require any modification when packages are made on a full manufacturing scale.

Integrity test methods for package processing and assembly validation studies are meant to verify the packages' continued conformance to the product's maximum allowable leakage limit. For some product–packages, the most fitting integrity test methods for this life cycle phase may have a detection limit greater than the maximum allowable leakage limit. Methods able to reject largely leaking packages such as those caused by defective or out-of-specification components, package damage, and/or package misassembly are appropriate. Seal quality tests suitable for use include those able to monitor package processing and/or assembly consistency.

Successful validation will result in a package that meets its user requirement specifications. The primary objective in package development and subsequent validation is to arrive at a quality product–package prepared using processes that reliably and consistently run within specified operating parameters as defined in the user requirement specifications, yielding critical package defects at a satisfactorily low rate. When performed, in-process and end-product package integrity testing should complement, not replace, thorough package development efforts.

2.2 Product Manufacturing

To ensure the quality of the manufactured product–package, it is critical to specify components of sufficient quality and to select vendors carefully. The factors to consider include:

- Acceptable results of the initial vendor or supplier evaluation
- Appropriate vendor acceptance quality limits and statistical sampling plan(s), or relevant certification
- Incoming component quality verification, including statistical assessment of quality against purchase specifications
- Appropriate procedure(s) for establishing corrective and preventive action when a vendor falls short of quality expectations

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Package integrity re-evaluation is considered when changes occur in the product, package design, package materials, or manufacturing/processing conditions. The extent of change control efforts are evaluated on a case-by-case basis, while the level of testing required to support the change is determined based on impact assessment.

The product's package integrity profile is compiled over the course of commercial manufacture. This database of ongoing leak and seal quality test results serves to flag potentially harmful package integrity excursions that may be linked to operative variations in package component design/material and package assembly/processing. Not all packaged-product damage can be readily detected post-assembly; therefore, processing procedure controls and monitors may be relied upon to safeguard against such problems. The proper combination of package leak tests, complementary package seal quality tests, and visual inspection is largely based on package failure results observed in earlier production validation and package development studies.

Testing production lot samples can provide a measure of package integrity confirmation, while entire testing by nondestructive means is able to yield an ongoing assessment of integrity assurance. In some cases regulatory requirement dictates the level of testing performed. For other product–packages, justification for the level of testing is based on statistical process control results generated during the validation phase, and later, on the basis of routine manufacturing product-quality trending analyses.

For example, glass or plastic ampules closed by heat fusion are customarily subjected to 100% nondestructive leak testing. Products sealed under vacuum require appropriate package assembly validation supplemented by testing over time to ensure that the vacuum is retained. Similarly, integrity assurance of packages that require a specific, non-reactive, inert gas headspace is based on appropriate package assembly validation along with testing for rise in reactive gas or water vapor content over time.

2.3 Commercial Product Stability

Container–closure integrity tests have been recommended as alternatives to sterility testing as part of commercial product stability programs. The goal is to ensure package integrity as a function of long-term product storage (*1*). [NOTE—Package integrity tests do not replace product release sterility tests.] Testing product for sterility is a poor measure of product–package integrity and also will not ensure product–package integrity over the shelf life of the product when performed as part of the stability program. In addition, a package may be in no danger of microbial ingress and yet be unable to maintain the gas headspace content necessary for product quality. Validated package integrity test methods using technologies such as those described in this chapter are more sensitive and reliable than product lot sterility testing for detecting a breach in package integrity that could lead to sterility loss or failure in relevant product physicochemical quality attributes.

Ideally, package integrity tests selected to support marketed-product stability studies are able to verify the absence of the smallest leaks of concern for a given product–package system. In other words, the product's maximum allowable leakage limit falls within the chosen test method leak detection range. However, the test methodology most appropriate for a particular product–package system may be unable to detect the very smallest leaks of concern. In some cases the package contents interfere with the ability of leak test methodologies to detect the smallest leaks. For example, proteinaceous ingredients or even salts may clog leak pathways, inhibiting leak detection by gas flow methods such as vacuum decay or mass extraction. It is prudent to understand the product's potential to interfere with the selected leak test method, both initially after package assembly and over time.

In situations such as those described above, the package integrity test chosen to support stability studies should have a detection limit as close as feasible to the product's maximum allowable leakage limit. An understanding of what the method is capable of evaluating and how this is applicable to microbial integrity assurance is warranted.

For products that demand package headspace content preservation, it is appropriate that the integrity test for stability studies verify the continued presence of specific headspace gases or subatmospheric pressure over time. Satisfactory results verify the absence of leaks that could jeopardize product sterility as well as relevant physicochemical quality attributes. The required duration for monitoring container vacuum or headspace gas content maintenance that equates to microbial barrier assurance and liquid product loss prevention can be predicted on the basis of gas flow kinetics, should a leak pathway exist that is large enough to allow microbial entry and product formulation loss.

Stability test samples intended for package integrity evaluation are kept at labeled storage conditions for the marketed product. Test sample quantities chosen for each testing time point are to be relevant and sufficiently representative of the purpose of stability testing, which is to indicate whether integrity is affected by the stability conditions. Sample quantity selection takes into account all prior development and validation testing.

As noted in reference (*1*), if an integrity test is nondestructive to the product or package, samples that pass package integrity testing may be further used in the stability testing for that specific test period or interval. However, samples should not be tested for package integrity at one time point (e.g., 12 months), then stored for further stability testing at a later time point (e.g., 24 months). While not specifically noted in reference (*1*), it is logical to assume that test samples earmarked for integrity verification over the course of the stability study could be checked for integrity before placement on stability if a nondestructive leak test method is used. This would be akin to the common practice of visually inspecting test samples prior to placing them on stability. In this way, subsequent integrity failures can be attributed to stability storage, rather than to other causes.

3. TEST METHOD SELECTION CRITERIA

No single package leak test or package seal quality test method is applicable to all product–package systems. Test method selection is made on a product–package on a case-by-case basis. Often more than one test method is employed during a given product's life cycle. Package test method selection as a function of product life-cycle phase, along with important integrity considerations, are discussed in *Package Integrity Testing in the Product Life Cycle*. A broad discussion of additional selection criteria for leak test methods follows. Specific leak test method examples are provided for informational purposes but are not intended as recommendations. The attributes and general capabilities of tests can be found in *Package Integrity Leak Test Technologies* ⟨1207.2⟩ and are helpful in the method selection process.

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3.1 Package Contents

When selecting a leak test method, the first determining factor is the nature of the package contents. Whether the package contains a liquid or solid formulation, and whether it contains a headspace of inert gas, air, vacuum, or even no headspace at all, will influence leak test method choice. For example, if testing liquid-filled packages by vacuum decay or mass extraction, test vacuum conditions may trigger some formulations to solidify inside leak paths, thereby blocking gas leakage flow and making the test ineffective. Alternatively, electrical conductivity and capacitance tests can be used, but only if the liquid product is more electrically conductive than the package materials.

3.2 Package Design, Materials of Construction, and Mechanics

Packages vary widely in their design, their materials of construction, and the mechanisms whereby package closure is affected. Each of these variables influences the leak test method choice, as the following examples illustrate.

3.2.1 RIGID OR FLEXIBLE PACKAGES

Rigid (nonporous) packages can tolerate the pressure or vacuum-challenge conditions required in several leak tests, including tracer liquid ingress, vacuum decay, mass extraction, and some tracer gas tests. In contrast, some flexible packages can tolerate differential-pressure test conditions only if special tooling is used to restrict package expansion and prevent subsequent seal damage. Package restriction is necessary to ensure consistent differential pressure conditions across the package barrier.

3.2.2 MOVEABLE OR FIXED COMPONENTS

Moveable package components, such as syringe stoppers (plungers or pistons), may require restraint to prevent their dislocation during the differential pressure test conditions required for a majority of leak test procedures (e.g., tracer liquid test, pressure or vacuum decay test, mass extraction test, bubble emission test, and microbial challenge by immersion test).

3.2.3 POLYMERIC MATERIALS

When exposed to test vacuum conditions, package materials such as plastics and some elastomers may outgas volatiles that raise vacuum-decay leak test results as well as mass extraction results, falsely implicating package leakage. Plastic packages that are highly permeable to tracer gases may not be compatible with helium tracer gas leak detection, as helium permeating through the package could be mistaken for package leakage or may mask small leaks. Special fixtures to limit tracer gas permeation effects and isolate tracer gas exposure to the seal area under test have been used to mitigate such difficulties.

3.2.4 METALLIC MATERIALS

Packages made of foil laminate materials may prove incompatible with electrical conductivity and capacitance leak detection, which works best with relatively nonconductive package materials. However, aluminum caps used to secure vials closed with elastomeric closures pose no hindrance to electrical conductivity and capacitance tests, even for finding leaks located between the closure and the vial finish.

3.2.5 TRANSPARENT OR OPAQUE MATERIALS

Packages made of transparent or translucent materials allow for visual inspection and electromagnetic wave passage. Therefore, transparent or translucent materials can be tested by laser-based gas headspace analysis techniques, as well as tracer-liquid ingress or microbial ingress. Opaque packages are incompatible with testing approaches that require visual inspection of the package contents.

3.3 Closure Type and Mechanics

Refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Closure Type and Mechanics* for a discussion of the closure type categories represented by the various product–package systems. The design of the closure system and its leakage restriction function (i.e., the maximum allowable leakage), plus the types of defects anticipated, influence the integrity test method choice.

3.4 Maximum Allowable Leakage Limit

The maximum allowable leakage limit as a function of the product–package quality requirement is discussed in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit*. The following integrity test method selection considerations are offered in light of that discussion.

3.4.1 STERILITY AND PRODUCT FORMULATION CONTENT MUST BE PRESERVED; GAS HEADSPACE PRESERVATION IS NOT REQUIRED

Integrity tests for this product quality category include those able to verify that the maximum allowable leakage limit that prevents liquid and microbial ingress is not exceeded. Tracer gas tests performed using the vacuum mode and laser-based gas headspace analysis test methods are two examples. Both have been shown to be sensitive enough to quantitatively analyze leakage through the smallest leak paths found to pose the smallest chance of liquid leakage or microbial ingress in rigid

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packaging. Such tests have also proven useful for defining relationships among package design, component fit, package assembly parameters, and leakage rate, even in the absence of package defects.

Leak testing of product-filled packages during later product-life-cycle phases often requires other tests. Leak test methods available for this phase of the product life cycle are able to be validated to reliably detect defects a few micrometers and larger. Examples of such physicochemical leak tests include vacuum or pressure-decay tests, mass extraction methods, electrical conductivity and capacitance tests, and liquid tracer tests.

3.4.2 STERILITY, PRODUCT FORMULATION CONTENT, AND GAS HEADSPACE CONTENT MUST BE PRESERVED

For this product quality category, leak test options include those that directly check for package headspace pressure and/or content, such as laser-based gas headspace analysis techniques. The detection limit for such methods is a function of the method's ability to accurately measure package headspace content or absolute pressure at the product acceptance limit, given the package headspace volume and the time lapse after package assembly. Such methods have broad application throughout the product life cycle.

3.4.3 STERILITY MUST BE PRESERVED; PRODUCT ACCESS IS REQUIRED

The product quality subcategory relates to the additional integrity requirement for those products that are contained in multiple-dose packages once the product is accessed by the end user. As discussed in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit, Sterility Must Be Preserved; Product Access is Required*, physicochemical as well as microbiological test methods designed to characterize and verify the unique barrier properties specific for the given container–closure system and its end-use application may be required (*2*).

Elastomeric closures of multiple-dose parenteral products are meant to reseal, limiting microbial ingress and product leakage during and between product access via needle puncture. The test in *Elastomeric Closures for Injection* ⟨381⟩, *Functionality Tests, Self-Sealing Capacity* is a blue dye liquid tracer test intended to screen closures for their ability to prevent gross liquid leakage post puncture. Caution is advised when solely relying on this test to prove a given closure's ability to reseal. Other test method(s) may need to be designed and implemented that more fully characterize the closure's leak prevention capabilities for the given product–package system and product end-use application.

3.5 Deterministic or Probabilistic Methods

A "deterministic leak test method" is one in which the leakage event is based on phenomena that follow a predictable chain of events, and leakage is measured using physicochemical technologies that are readily controlled and monitored, yielding objective quantitative data. Most deterministic leak test methods rely on the predictable movement of gas that inevitably occurs through an open leak path, given specific differential pressure and/or partial pressure test conditions (e.g., tracer gas, laser-based gas headspace analysis, pressure decay, vacuum decay, and mass extraction). The electrical conductivity and capacitance test is a deterministic leak test approach that relies on the more predictable presence of liquid near a leak path, rather than the less predictable movement of liquid through a leak. Deterministic methods are characterized as being capable of reproducibly detecting leaks at clearly defined and predictable detection limits. Because the majority of deterministic leak test methods described in this chapter require no special test sample preparation, sample preparation error is eliminated.

A "probabilistic leak test method" is stochastic in nature in that it relies on a series of sequential and/or simultaneous events each associated with uncertainties, yielding random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate larger sample sizes and rigorous test condition controls to obtain meaningful results. Typically, sample size and test condition rigor are inversely related to leak size. Therefore probabilistic leak test methods are more challenging to design, develop, validate, and implement, especially when used to find leaks near the upper and lower limits of the test method's detection range. Probabilistic methods include microbial challenge tests, as well as some physicochemical tests. These methods include bubble emission tests, tracer liquid tests (employing either qualitative or quantitative measurement methods), and tracer gas tests by the sniffer probe approach.

A deterministic leak test method having the ability to detect leaks at the product's maximum allowable leakage limit is preferred when establishing the inherent integrity of a container–closure system. Deterministic methods may also be chosen if test sample quantities are limited, when checking for rarely occurring leaks of concern and/or when the potential risk for failing to find leaks of a given size or type is too great. Probabilistic methods are best chosen when the method outcome requirements demand a specific probabilistic testing approach. Probabilistic tests are further discussed in *Method Outcome* and *Quantitative or Qualitative Methods of Analysis* below.

3.6 Physicochemical or Microbiological Methods

"Physicochemical leak test methods" are those that use physical and/or chemical analysis techniques to test for package integrity. Physicochemical methods include both deterministic and probabilistic leak test methods. "Microbiological leak test methods" are probabilistic methods of analysis that use viable microorganisms to evaluate test sample integrity. This chapter includes the microbial challenge test by immersion exposure.

The following section includes additional information on the test method outcome criterion that is related to the microbiological challenge test method.

3.7 Method Outcome

The outcome(s) sought from leak test results will often drive the test method choice. These desired outcomes may include:

- Leak path presence of detection

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- Leak path location determination
- Leakage rate measurement
- Liquid egress/ingress potential
- Microbial ingress potential

All leak test methods are meant to identify the leak path presence. Often, leak tests are able to provide additional outcome information. Methods that also provide evidence of leak path location include electrical conductivity and capacitance tests, bubble emission tests, tracer gas sniffer probe tests, and some tracer liquid tests. Methods that provide a measure of whole-package leakage rate include laser-based gas headspace analysis, mass extraction, pressure and vacuum decay methods, and tracer gas tests by the vacuum mode.

Microbial challenge tests provide information on the degree of protection afforded by the product–package against microbial ingress that occurs via active growth or motility through leak pathways and/or by liquid carrier passive transport through leak pathways. Microbiological challenge tests help to clarify the risks to product sterility posed by specific package materials, package designs, or potential package barrier breaches. Sterility risks linked to particular environmental exposure or product use conditions may also demand a microbiological challenge methodology. In summary, all leak test methods at minimum detect leaks; some methods may provide more information, but no single method alone can yield all four outcomes listed above.

3.8 Quantitative or Qualitative Methods of Analysis

The measure of analysis can play a part in leak test method selection. In other words, does the method yield quantitative data that allow for objective analysis, or are the data strictly qualitative and require more subjective interpretation? Leak test methods that use a "quantitative measure of analysis" include electrical conductivity and capacitance tests, laser-based gas headspace analysis, mass extraction, pressure and vacuum decay, tracer gas tests (especially when testing via the vacuum mode), and tracer liquid tests that use quantitative analysis (e.g., spectrophotometric analysis).

Conversely, a "qualitative measure of analysis" is based on subjective observation of a specific quality, attribute, or characteristic of the test sample, e.g., a visual check for turbidity when evaluating microbial challenge test samples. Bubble emission tests that report visible evidence of continuous bubbling, and tracer liquid tests that rely on visible evidence of dye migration are other examples of subjective and qualitative analysis. Because qualitative measurement results are subject to interpretation, they may be prone to human error. When method considerations permit, leak test methods that yield quantitative measurements are preferred.

3.9 Leak Test Detection Limit

The detection limit of a leak test is the smallest leakage rate or leak size that the method can reliably detect, given the product–package of interest. A large variety of measurement units are used to describe leakage rates and leak sizes when specifying the detection limits (and detection ranges) of leak test methods. This often leads to confusion when comparing the performance claims of various instrument manufacturers or examining test results generated by multiple methods.

To address this, *Table 1* presents the relationship between orifice size (assuming a perfect hole of negligible length) and the rate at which dry air would pass through such a hole when exposed to 1 atmosphere (atm) differential pressure at a specified temperature.

These leakage rates and leak sizes are theoretical approximations and are not definitive.

Table 1 services two purposes. First, it is meant to help the reader better grasp the relationship between theoretical hole diameter and the gas leakage rate. Second, it provides a common measurement scale that can be referred to later in this chapter to more simply state leak detection limits for the various technologies described. For example, test technology X found in published studies to detect leaks as small as about 8 µm would be referred to in ⟨1207.2⟩ as having an approximate leak detection limit of row 4 in *Table 1*.

Leak detection limit should not be the only or perhaps even the primary basis for choosing a test method. Often, the best method for a given application is dictated by other factors. For example, a tracer gas leak test method having an extremely small leakage rate detection limit may be the proper choice for establishing the inherent package integrity of a stoppered glass vial as a function of capping machine parameters during package development. Yet this method would be an inappropriate choice for rapid on-line testing in routine manufacturing. Instead, an electrical conductivity and capacitance test with a larger leak detection limit, able to test product-filled packages at on-line speeds, may be the best option. Refer to *Detection Limit* for a discussion on how to determine the limit of detection.

Table 1. Gaseous Leakage Rate versus Orifice Leak Size[a]

Row	Air Leakage Rate[b] (std · cm^3/s)	Orifice Leak Size[c] (µm)
1	$<1.4 \times 10^{-6}$	<0.1
2	1.4×10^{-6} to 1.4×10^{-4}	0.1 to 1.0
3	$>1.4 \times 10^{-4}$ to 3.6×10^{-3}	>1.0 to 5.0
4	$>3.6 \times 10^{-3}$ to 1.4×10^{-2}	>5.0 to 10.0
5	$>1.4 \times 10^{-2}$ to 0.36	>10.0 to 50.0

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Table 1. Gaseous Leakage Rate versus Orifice Leak Size[a] *(continued)*

Row	Air Leakage Rate[b] (std · cm³/s)	Orifice Leak Size[c] (µm)
6	>0.36	>50.0

[a] This table is not intended for ranking test methods but is offered as an aid for expressing test method leak detection capabilities in this chapter.
[b] Dry air leakage rate measured at 1 atm differential pressure across an orifice leak (i.e., leak inlet pressure of 1 atm versus outlet pressure of approximately 1 Torr) at 25°. The theoretical correlations of orifice sizes to air leakage rates were provided by Lenox Laser, Glen Arm, MD. Leakage rates are approximation ranges.
[c] Nominal diameter orifice sizes assume a leak path of negligible length. Orifice sizes are approximation ranges.

3.10 Leak Test Method Range

Leak test method range is the interval between the smallest and largest leak size (or leakage rate) that can be detected by a given leak test method with a suitable level of accuracy and precision. All leak test methods have an optimum detection range. Therefore, it is possible that additional tests may be needed to catch those leaks of concern that fall outside the chosen leak test method's detection range. For example, a test method able to find the smallest leaks may miss gross leakage such as a missing package component. Vision systems that check for major package defects such as a missing package component or a package crack may be required. Finally, before using a test method to check for large leaks, the impact of gross defects on leak test instrumentation should be considered; some instruments may malfunction or become damaged upon exposure to a leaking product or damaged packages. Refer to *Range* for additional detection range information.

3.11 Nondestructive or Destructive Methods

The need to preserve the test product–packages may influence the decision to select a nondestructive leak test method, rather than a destructive one. Destructive test methods damage the test sample and/or expose it to potential contaminants; hence, the product is not recoverable. Only nondestructive test methods are appropriate for leak testing product–package units earmarked for commercial or clinical study distribution.

Examples of destructive leak test methods include tracer liquid ingress tests, bubble emission tests, and microbiological challenge tests. Tracer gas and pressure decay leak tests are considered destructive if they require that the package barrier be compromised in order to introduce tracer or pressurizing gas into the assembled package. Examples of nondestructive leak test methods include mass extraction and vacuum decay leak tests, as well as noninvasive gas headspace analysis tests. Electrical conductivity and capacitance leak tests are deemed nondestructive if it can be shown that electrical current exposure causes no harm to the product; in rare instances, exposure has triggered headspace ozone formation, causing product oxidation (*3*).

3.12 Off-Line or On-Line Methods

Off-line leak testing is generally performed on a stratified random sampling of the product lot, away from the manufacturing line. Off-line package evaluation allows for the use of any validated nondestructive or destructive leak test and seal quality test option compatible with the product–package. Off-line testing can accommodate slower test cycle times; for methods in which test time is a performance factor, a slower off-line test is often more sensitive than its on-line counterpart. Off-line tests typically are less costly to perform as they utilize bench-top or smaller scale equipment without the sophisticated product-handling machinery required to support higher speed on-line processes.

On-line leak testing is commonly integrated into a continuous fill and seal product–package manufacturing process. A prerequisite for an on-line leak test method for entire lot testing is that it be nondestructive to the package and its contents. On-line testing can potentially provide greater assurance that all packages have integrity and can yield instant feedback in the event of package misassembly or breakage, enabling real-time line corrections. For some test systems, incorporating large-scale leak detection equipment into a complex high-speed product–package filling and assembly line can be prohibitive. Higher line speeds leading to shorter leak detection test cycle times can limit test method detection capability. In addition, the impact of instrument downtime on the production run as a result of leaking packages or possible equipment malfunction is an important consideration. A separate leak testing line may be set up outside the sterile manufacturing suite to allow for full lot testing without the complications of leak test integration with package filling/sealing operations. A few examples of on-line leak test technologies include electrical conductivity and capacitance, vacuum decay leak, and noninvasive laser-based gas headspace analysis tests.

4. TEST INSTRUMENT QUALIFICATION, METHOD DEVELOPMENT, AND METHOD VALIDATION

Leak test methods are validated in order to demonstrate method effectiveness. Method validation is preceded by instrument/equipment qualification, followed by test method development. The following discussion specific to leak test methods is intended to supplement the guidance for analytical instrument qualification presented in *Analytical Instrument Qualification* ⟨1058⟩, plus the guidance for method validation provided in *Validation of Compendial Procedures* ⟨1225⟩.

4.1 Instrumentation and Equipment Qualification

The qualification of instruments or equipment to be used for leak testing includes: 1) evaluation of instrument/equipment functionality, and 2) determination of test system detection capabilities using appropriate calibration tools or reference standards to simulate with-leak test conditions.

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4.2 Method Development and Validation

After successful instrument/equipment qualification, leak test method parameters are developed and optimized to ensure a leak test method is able to meet all relevant leak detection performance criteria specific for the test product–package system. The following properties of a valid test method are defined as they relate to package integrity tests.

4.2.1 ACCURACY

"Accuracy" is a measure of the method's ability to correctly differentiate packages that leak above the claimed detection limit from those that leak below this limit (i.e., do not leak). Accuracy provides a measure of false positive and false negative occurrence. Alternatively, for those methods that deliver an outcome that is a direct quantitative measure of gas leakage rate (or of gas concentration or gas pressure), accuracy is a measure of the method's ability to produce an outcome comparable to a true standard. For example, helium mass spectrometry provides a direct measure of helium leakage rate. Accuracy is the closeness of the instrument reading to the certified leakage rate of a nationally recognized traceable standard.

4.2.2 PRECISION

"Precision" is the ability of the method to yield reliable, repeatable data. Precision includes repeatability (e.g., repeat testing of a homogeneous test sample population), ruggedness (within laboratory tests performed, for example, by multiple operators on multiple days, using multiple instruments; also known as intermediate precision), and reproducibility (among laboratories tests). The level of precision to which a leak test method is validated is often a function of resource availability (e.g., one instrument versus multiple instruments) and intended test method application (e.g., use of the method at one test site only versus across multiple test sites).

4.2.3 SPECIFICITY

"Specificity" is the ability of the method to accurately differentiate between leaking and nonleaking packages, despite interfering factors that may cause false detection. For example, when employing tracer gas leak detection using helium mass spectrometry (vacuum mode), excessive helium permeation through the package wall may mask small package leaks or may be falsely interpreted as leakage in no-defect packages.

4.2.4 DETECTION LIMIT

Refer to *Leak Test Detection Limit* for an introduction to this topic. The detection limit of a leak test is specific for a given testing approach when performed using a specific instrument make/model in evaluating a given product–package system. Utilizing the principles described in ⟨1225⟩, detection limit is demonstrated by challenging packages with and without known defects by the leak test method for multiple test days by multiple operators. The intended application of the method will dictate the level of precision required (i.e., whether to incorporate multiple operators/instruments/laboratories, etc.).

A common challenge with small leak detection is the potential for interfering factors to be misconstrued as leakage presence. For example, vacuum decay leak tests measure the rise in pressure inside an evacuated chamber containing the test package. Package leakage causes chamber pressure to rise, but package material volatiles, test system moisture, and package expansion may also do the same. Gas permeating through a package wall detected by a tracer gas test may be mistaken for leakage. Bubbles emitted during a bubble test may actually be the result of package surface outgassing, volatilization of dissolved gases in the immersion fluid, or the release of trapped air between package components.

The absence of a leak detection signal may also be misinterpreted as the absence of leaks. For example, tracer liquid tests may fail to reliably detect small leaks due to any one of a number of factors including air locks, product, or debris in the leak path; liquid surface tension; leak path geometry; or insufficient differential pressure test conditions. The same is true for microbial ingress tests that are further subject to the inherent variability of living microorganisms.

In short, false negative results that miss leaks and false positive results that incorrectly suggest leak presence are possible with any leak test method. Therefore, test method detection limit is determined by comparing readings of intentionally defective packages to nondefective ones. Leak detection limit determination test units consist of a randomly ordered population mix of negative and positive control units (refer to *Negative and Positive Controls*). Nondestructive test methods can employ the same set of units for multiple test exposures, while destructive methods will require a new set for each test series. Control subset unit quantities are chosen based on several factors: 1) the deterministic or probabilistic nature of the outcome, 2) the inherent package-to-package variability that may influence test results, and 3) the statistical confidence level required by the test acceptance criterion. The positive control subset includes units with defects sized to the anticipated detection limit, in addition to units with leaks bracketing this size limit. If the detection range is to be established, controls having large defects are included. Positive controls representing a wide defect size range are especially important for probabilistic methods in order to clearly understand leak detection likelihood as a function of leak size.

Because of the many product–packages and leak-testing options available, the resultant leak detection limit is more meaningfully stated when the negative and positive control subsets used, the test precision level, and the test results are summarized. The following is an example of expressing a test method's limit of detection:

"The detection limit for method X was determined to be 5 ± 2 µm. Validation studies found defects of this nominal size were detected 95% of the time; all larger defects were detected 100% of the time. Studies included three replicate test series performed on multiple days by multiple operators in a single laboratory using one instrument. Detection limit was determined using product-filled packages. Test units in each series included a negative control subset of 300 units (each without defect) and a positive control subset of 90 units (each having a laser-drilled defect ranging in nominal size from 1.5 ± 0.6 µm to 15 ± 3 µm). Each defect was independently size-certified by comparing the dry air leakage rate at 1 atm differential pressure (leak inlet pressure of 1 atm versus outlet pressure of approximately 1 Torr) at 25° to that of standard orifice leaks."

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[NOTE—This is one example of how test method detection limit could be expressed and is not to be considered compulsory or restrictive either in content or level of detail.]

Leak test technologies exist that are able to detect leaks even smaller than can be artificially created in a positive control test set. Two examples follow. In both cases, a limited number of positive controls can serve to verify that the instrument set-up is able to detect leaks of specific type and in specific package locations but not to determine leak size detection limit capability.

- Tracer gas tests by helium mass spectrometry performed in the vacuum mode can detect leaks as small as about 10^{-11} mbar · L/s, which is about 5 logarithmic leakage units smaller than leakage through a hole 0.2 µm in size. For such methods, leakage rate detection ability can only be verified by using nationally recognized gas leakage rate standards to introduce leaks into the test system. Because gas permeation can be mistaken for leaks, it is important to experimentally establish the relationship between tracer gas leakage rate and permeation rate as a function of test cycle time for a given package system.
- Laser-based gas headspace analysis is another approach that may be able to identify the presence of leaks smaller than can be artificially created. For such methods, the limit of detection can be mathematically predicted on the basis of gas flow kinetics and is a function of the time lapse between analyses, and the smallest gas content or pressure change that can be reliably detected by the instrument for the given package system.

4.2.5 QUANTITATION LIMIT

"Quantitation limit" is that lowest leakage rate or leak size that can be determined with acceptable accuracy and precision under the stated experimental conditions. Laser-based gas headspace analysis is a method that may be evaluated for quantitation limit. For most leak test methods, detection limit is more meaningful.

4.2.6 LINEARITY

"Linearity" is the ability of the method to elicit test results that are mathematically proportional to leak path size or leakage rate. Deterministic leak test methods that exhibit linearity include laser-based gas headspace analysis and tracer gas analysis (vacuum mode). Other methods such as vacuum decay, pressure decay, and mass extraction also produce results that correlate to leak size or leakage rate. However, test findings are generally intended to identify leak presence and perhaps to understand relative leak size; they are not typically relied upon for leak size or leakage rate quantitation. Electrical conductivity and capacitance tests and all referenced probabilistic methods are not validated for linearity.

4.2.7 RANGE

Leak detection range is defined in *Leak Test Method Range*. Leak test method range is explored in test method development to better understand leak test method detection limitations. For methods being relied upon to detect leaks within a specified size range, detection at the upper range limit may be confirmed in validation. Range is evaluated by using sets of negative controls and appropriately sized larger-defect positive controls. The large-defect positive control subset may include defects of various types likely to occur for the given product-package system (refer to *Type Defects*).

4.2.8 ROBUSTNESS

"Robustness" is the method's ability to accurately identify leaking versus nonleaking packages despite small but deliberate variations in procedural parameters, providing an indication of the method's suitability during normal usage. One way to evaluate robustness is to perform the test using test parameters bracketing optimal or normal test specifications. Parameters to be varied are those having the greatest impact on test results; variation should reflect instrument performance accuracy. For example, the robustness of a vacuum decay leak test with a test cycle time of 30 s (accurate to within 0.5 s) might be demonstrated during method development by verifying method performance at set test cycle times of 29.5 and 30.5 s.

4.3 System Suitability

One outcome of test method development and validation is the establishment of a system suitability test(s), also called a performance verification test(s). "System suitability" is a manner of ensuring that the leak test method, including all factors that may be subject to variability that may impact test results (e.g., instrumentation, analysts, test sample preparation, and the test environment), is adequately controlled and maintained in such fashion that the method is rugged and robust. System suitability is important for all leak test methods. Extra care may be required for probabilistic methods more prone to variability.

For example, a highly instrumental method such as a mass extraction test may be checked for system suitability by leak testing a master package unit both with and without the added challenge of external air introduced to the test system via a calibrated leak adjusted to the method's limit of detection. If the method requires preliminary test package preparation (e.g., a drying step), then it would be appropriate to demonstrate that an intact package prepared in the prescribed manner elicits the anticipated mass flow rate response. System suitability can be performed at the beginning and end of each testing sequence for added method assurance.

System suitability for a test more probabilistic in nature takes into account multiple test method variables and may require greater numbers of challenge samples (i.e., positive and negative controls) for adequate method assurance. For example, tracer gas tests via a sniffer probe may require a routine demonstration that the operator is able to successfully differentiate packages without defect from those with leaks (ranging in size from smallest to largest, located at various package positions) in a blinded challenge study. Alternatively, the operator could be challenged by randomly introducing defective samples (unknown to the inspector) among the test sample population during routine test procedures.

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4.4 Microbial Ingress Risk Comparison

Appropriate leak test method validation practices mirror compendial analytical test validation guidelines. In the past, validation of physicochemical leak test methods used for pharmaceutical sterile product packages also routinely included a direct or indirect comparison of physicochemical leak test results to microbiological challenge data. While an understanding of the relationship between physicochemical leak test method capabilities and microbial ingress risk may be important, an experimental comparison may not be needed or useful in all cases. [NOTE—The reader may refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit* for a discussion of maximum allowable leakage limit and inherent package integrity.]

The following situations illustrate when a comparison study of the microbial ingress or liquid leakage risk to physicochemical leak test method capability relationship is likely not needed. [NOTE—This is not intended to be a complete or exhaustive listing of all situations for which comparison studies would not be required or useful but is provided for illustration only.]

- If the validated physicochemical leak test method has a proven detection limit at or below the product-package maximum allowable leakage limit.
- If the validated physicochemical leak test method is not being used to verify the absence of all leaks of concern. Instead, the method is being used to find leaks notably larger than the maximum allowable leakage limit. For example, a rapid on-line test shown to reliably detect leaks of 25–150 µm in nominal diameter is being used to reject damaged or misassembled product-filled packages within the method's leak detection range.

The following situations illustrate when an experimental indirect or direct comparison of the microbial ingress risk (or liquid leakage risk) to physicochemical leak test method capability relationship may be useful. [NOTE—This is not intended to be a complete or exhaustive listing of all situations for which comparison studies could prove beneficial but is provided for illustration only. These examples are not compulsory but are provided for instruction only.]

- If the validated physicochemical leak test method is being relied upon to measure or confirm the inherent package integrity of a product–package system, but the method's limit of detection is notably greater than the maximum allowable leakage limit. For example, a package has a maximum allowable leakage limit of less than 6×10^{-6} mbar · L/s as measured by helium mass spectrometry (equivalent to holes less than approximately 0.2 ± 0.1 µm in nominal diameter). This limit was chosen based on published literature references. But the leak test method of choice for testing product-filled packages placed on stability is able to detect leaks equivalent to holes 3 µm in nominal diameter and larger. A study correlating microbial ingress or liquid leakage risk to defect type/size can provide a measure of the likelihood of microbial ingress (and/or liquid leakage) at the leak test method detection limit, and thus can provide an understanding of ability of the stability leak test to identify leaks of concern.
- If the validated physicochemical leak test method is being used to measure or verify the inherent package integrity of a product–package system, but the maximum allowable leakage limit is either lacking or not well defined. For example, a unique package is being used for which the maximum allowable leakage limit that will ensure absence of product loss or microbial ingress has not been determined. A study comparing the risk of microbial ingress or liquid leakage to leak type/size, and in turn to the likelihood of detection by the physicochemical leak test method may be useful.

When performing microbial ingress risk assessment studies it is important to keep in mind the probabilistic nature of microbial ingress. To achieve the most meaningful data, large population sets of negative and positive controls should be used. Test protocols should be thorough and well designed, taking into consideration the multiple factors and variables that can influence results. As suggested above, liquid leakage risk assessment studies may substitute for microbial ingress risk studies assuming the risk of liquid leakage is equivalent to or greater than that of microbial ingress, with appropriate justification.

4.5 Negative and Positive Controls

"Negative controls" are packages with no known leak, and "positive controls" are packages with intentional or known leaks. Negative and positive controls are designed and assembled for use in method development and validation with consideration given to container–closure design, materials of construction, characteristics of anticipated package leaks, and impact of product contents on test results. Negative and positive controls should represent packages assembled in a typical manner as the product being tested using normally processed components, the exception being the intentionally created leak in each unit of the positive control subset. Some leak test methods may necessitate positive controls that simulate test product headspace and formulation contents as well. System suitability checks for some test methods employ negative and positive controls (refer to *System Suitability*).

Test blanks should not be confused with negative controls. For example, liquid tracer leak detection by spectrophotometric analysis may require a blank solution without the liquid tracer element to confirm instrument baseline performance.

A "master" is a type of negative control test unit. It is a package prototype, model, or facsimile made to simulate the test package in shape and design. Masters may be made of solid material such as plastic or metal, or they may be simply a designated container–closure unit. Masters are no-leaking mock packages often used in system suitability verification tests for leak tests to verify instrument performance, such as for vacuum decay or mass extraction testing.

4.5.1 DEFECT CREATION METHODS

The positive control set typically represents a range of package defect sizes and types. Numerous approaches have been used to create package defects. When creating positive controls, a fundamental awareness of leakage dynamics as a function of different defect types and materials of construction is important.

Placing a so-called "hole" or break in the package wall is one positive control creation approach. In this case the defect materials of construction are identical to the package itself, thus potential test method interference due to product exposure to the package material can be readily identified. An example of such interference is the clogging of small leak paths by product formulation observed during vacuum decay, mass extraction, or tracer gas (vacuum mode) tests.

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Laser drilling is often used to create package defects commonly referred to as holes. However, these so-called holes are not pristine orifices but are non-cylindrical and asymmetric and may consist of a tortuous matrix of micro-cracks. While laser-drilled defects are not dimensionally ideal, they offer the advantages of closely simulating actual package defects (e.g., cracks) and do not require the introduction of foreign materials, such as wires, tubes, or epoxies, that may influence leakage dynamics. Laser-drilled defects are often sized for nominal diameter by comparing dry air leakage rate through the defect at specified conditions of pressure and temperature to flow rates through standard orifice leaks in thin metal plates. Currently, laser-drilled defects in rigid glass or plastic components can be made as small as about 2–3 µm in nominal diameter and to about 5–10 µm in nominal diameter in flexible thicker wall package materials. Smaller diameter defects tend to clog from handling, environmental debris, or flexible package wall deflection.

Glass micropipettes can be used to simulate single-orifice defects as small as about 0.1 µm in diameter. The tip diameter can be nominally sized using air flow measurements. When creating positive controls, micropipettes are inserted through a break in the package wall, then an appropriate sealant is applied to the insertion site. Challenges to micropipette use include ensuring a complete seal between the micropipette perimeter and the package wall and avoiding micropipette tip damage.

In addition, air trapped in the pipette tube can interfere with leak test methods that depend on fluid flow through the leak path, such as tracer liquid tests and microbial challenge ingress tests.

Microtubes (also called microcapillaries) inserted through the package wall and fixed in place with sealant are another means of creating positive control defects. Microtubes can be made of a variety of materials, can be cut to any length and can be as narrow as 2 µm in cross-sectional diameter. Microtubes are often employed as a substitute for a smaller-bore, shorter-length leak path when performing leak tests that rely on gas flow measurements. However, caution is advised before choosing lengthy, larger-bore microtubes to simulate an orifice leak of smaller diameter for leak tests that rely on the passage of liquids or microorganisms. Fluid dynamic theory correlating fluid flow through capillary tubes to pass through smaller bore holes is based on the unimpeded passage of ideal liquids through capillaries at equilibrium pressure conditions. Liquid product formulation and aqueous media flow into and through a microtube is complicated by numerous factors including liquid surface tension, liquid viscosity, surface contact angle, airlocks, particulate blockage, and tube-wall and tube-end finishes. Microbial ingress through microtube defects relies more on the presence of liquid in the tube than on the physical barrier to passage or grow-through afforded by the tube diameter (*4*). Microtubes are a logical choice when creating defects representing channel defects. In this case microtube length should mimic as closely as possible the actual package barrier thickness (package wall or seal width). Microtube use challenges include effecting smooth, perpendicular cuts of microtube ends and adequately sealing microtubes into the test sample wall.

Other commonly used defect creation methods include inserting a needle through the package wall; placing a wire, microfilament, or film between sealing surfaces; and adhering a holed, thin metal plate onto package surfaces. It is important to note that defects made by using an object foreign to the package (e.g., needle, film, wire plate) may display gas, liquid, or microbial leakage dynamics markedly different from that of actual defects (*5*). Positive controls made by such means are easy and inexpensive approaches for creating larger size defects useful for test method feasibility studies and for exploring a test method's detection range upper limit.

4.5.2 TYPE DEFECTS

"Type defects" are positive controls representing realistic package flaws. Type defects are important to include in method validation studies to explore a leak test method's practical application in detecting realistic package failures and flaws. A few types of defect examples are listed below:

- Heat sealed bag: 1) weak seal, 2) wrinkled seal, 3) seal gap, 4) seal channel, 5) product entrapment in seal
- Stoppered vial package: 1) vial finish channel defect, 2) loosely capped stopper, 3) product trapped between stopper and vial
- Prefilled syringe: 1) needle shield punctured by staked needle, 2) defective plunger
- Ophthalmic dropper bottle: 1) loose cap, 2) missing or poorly inserted dropper tip, 3) defective tip or cap

Assigning precise sizes to type defects is generally not meaningful or feasible because of their inherent irregularity and complexity. Type defects are defined in more qualitative, descriptive terms such as those listed above. Because no leak test method can find all possible defects in a given container–closure system, information collected from type defect tests can be used to identify alternative approaches to detecting or limiting the occurrence of critical imperfections not readily found by the chosen leak test method.

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⟨1207.2⟩ PACKAGE INTEGRITY LEAK TEST TECHNOLOGIES

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1. INTRODUCTION

The purpose of this chapter is to provide information guiding the selection and proper use of leak test technologies (also called methodologies, approaches, or methods). The leak test technologies described in this chapter were selected on the basis of relevant research study data published in peer-reviewed journals and/or precision and bias study data generated in support of recognized test method standards. When referencing standard test methods (e.g., ASTM), the reader is advised to refer to the most recent versions. In some cases, the scope of referenced standard test methods does not include the package types of the scope in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩. In all cases, methods and literature studies are cited to provide benchmark information useful for pharmaceutical package leak test method development and validation.

The technologies described in this chapter are not prescriptive methods but represent testing concepts that may be applied when leak testing sterile product–packages. Test technologies vary in terms of their potential detection limits, reliability, and applications; therefore, none are universally appropriate for leak testing all product–packages. This chapter provides information to allow a thorough comparison of testing approaches so that the most appropriate technology for a given situation can be identified.

After a methodology has been selected for use, the test equipment operation and performance is qualified. Test method parameters are optimized during method development and confirmed during validation. Thus, a final leak test method is specific to a particular container–closure or product–package system.

The leak test methods included are divided into two categories: deterministic and probabilistic. Deterministic leak test methods (*Table 1*) are preferred over probabilistic methods when other key method selection criteria permit. Probabilistic leak test methods (*Table 2*) are best used when the product–package system proves incompatible with deterministic methods, or when method outcome requirements demand a particular probabilistic testing approach.

In this chapter's *Table 1* and *Table 2*, the "leak size detection limit" provided for each methodology refers to leakage rates/leak sizes listed by row in *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩, *Table 1*. The reported leak detection limits were chosen on the basis of literature sources, as well as commonly accepted experience. This information is intended to aid in the selection of the test technology but should not be used as a definitive statement of test method performance for any specific leak test method applied to any given product–package system. Instead, leak detection limit and range should be established during leak test method development and validation for the respective product–package or container–closure system. For instance, an approach cited as capable of detecting row 6 leaks may be validated by the user to detect leaks as small as those in row 3. Conversely, a method described as capable of detecting leaks in row 3 may be determined by the user to detect leaks only as small as row 5.

This battery of testing technologies and the information provided are intended to aid, not limit, the selection, development, validation, and use of leak test methods. Unlisted methodologies shown by the user to meet the qualification and validation requirements for a satisfactory leak test may be used. In addition, listed technologies may demonstrate expanded testing capabilities beyond those currently identified.

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Table 1. Deterministic Leak Test Technologies[a]

Deterministic Leak Test Technologies	Package Content Requirements	Package Requirements	Leak Detection Limit[b]	Measurement Outcome and Data Analysis	Effect of Method on Package	Test Time Order of Magnitude
Electrical conductivity and capacitance (high-voltage leak detection)	Liquid (with no combustion risk) must be more electrically conductive than package. Product must be present at leak site.	Less electrically conductive than liquid product.	**Row 3** Varies with product–package, instrument, test sample fixtures, and method parameters.	Quantitative measure of electrical current passing through the test sample; provides an indirect determination of leak presence and leak location as shown by a drop in test sample electrical resistivity, with a resultant increase in voltage reading above a predetermined pass/fail limit.	Nondestructive, although impact of test exposure on product stability is recommended	Seconds
Laser-based gas headspace analysis	Gas volume, path length, and content must be compatible with instrument's detection capability.	Allows transmission of near-IR light.	**Row 1** Varies as a function of time span between analyses.	Quantitative measure of gas headspace content of the test sample by laser-based gas analysis, for a product requiring a headspace low in oxygen, carbon dioxide, or water vapor concentration; and/or low in absolute pressure. Whole test sample leakage rate is determined by compiling readings as a function of time.	Nondestructive	Seconds
Mass extraction	Gas or liquid must be present at leak site. Presence of liquid at leak site requires test pressures below vapor pressure. Product must not clog leak path.	Rigid, or flexible with package restraint mechanism.	**Row 3** Varies with product–package, instrument, test fixtures/chamber, and method parameters.	Quantitative measure of mass flow rate resulting from test sample headspace escape or liquid product volatilization within an evacuated test chamber housing the test sample. Quantitative pressure readings early in the test cycle indicate larger leak presence. Whole test sample leakage rate is determined by comparing the test sample mass flow results to results using leak rate standards and positive controls.	Nondestructive	Seconds to minutes
Pressure decay	Gas must be present at leak site. Product (especially liquids or semi-solids) must not cover potential leak sites.	Compatible with pressure detection mode. Rigid, or flexible with package restraint mechanism.	**Row 3** Varies with product–package, instrument, and method parameters.	Quantitative measure of pressure drop within a pressurized test sample. Pressure drop readings are a measure of gas escape through leak paths. Whole test sample leakage rate is determined by comparing pressure decay results to results using leak rate standards and positive controls.	Nondestructive, unless the means used to access test sample interior compromises test sample barrier.	Minutes to days, depending on package volume and required leak limit of detection
Tracer gas detection, vacuum mode	Tracer gas must be added to package. Tracer gas must have access to package surfaces being tested for leaks.	Able to tolerate high-vacuum test conditions Rigid, or flexible with package restraint mechanism Limited tracer gas permeability	**Row 1** Varies with instrument capability and test sample fixtures.	Quantitative measure by spectroscopic analysis of tracer gas leak rate emitted from a tracer-flooded test sample positioned in an evacuated test chamber. Whole test sample leakage rate is calculated by normalizing the measured tracer leak rate by tracer concentration in the test sample.	Nondestructive, unless tracer gas introduction into the package compromises test sample barrier.	Seconds to minutes
Vacuum decay	Gas or liquid must be present at leak site. Presence of liquid at leak site requires test pressures below vapor pressure. Product must not clog leak path.	Rigid, or flexible with package restraint mechanism	**Row 3** Varies with product–package, instrument, test sample chamber, and method parameters.	Quantitative measure of pressure rise (vacuum decay) within an evacuated test chamber housing the test sample; vacuum decay readings are a measure of headspace escape from the test sample, or liquid product volatilization. Whole test sample leakage rate is determined by comparing vacuum decay results for the test sample to results of tests performed using leak rate standards and positive controls.	Nondestructive	Seconds to minutes

[a] All methods apply to nonporous, rigid and flexible packages as per the scope of ⟨1207⟩.

[b] The leak detection limit cited for each technology refers to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Table 1* and is provided for information only. This information is intended to assist in early methodology selection. The validated leak detection limit for a product–package test method may deviate from these values.

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Table 2. Probabilistic Leak Test Technologies[a]

Probabilistic Leak Test Technologies	Package Content Requirements	Package Requirements	Leak Detection Limit[b]	Measurement Outcome and Data Analysis	Effect of Method on Package	Test Time Order of Magnitude
Bubble emission	Gas must be present at leak site. Product (especially liquids or semi-solids) must not cover package surfaces to be leak tested.	Rigid, or flexible with package restraint mechanism.	**Row 4** Varies with product–package, test sample fixtures and positioning, method parameters, and analyst technique and skill.	Qualitative measure by visual inspection of bubble emission caused by escape of test sample headspace while sample is submerged and exposed to differential pressure conditions. Alternatively, sample surfaces may be exposed to surfactant. Continuous bubble emission indicates leak presence, location, and relative size.	Destructive	Minutes
Microbial challenge, immersion exposure	Growth-supportive media or product. Presence of liquid at the leak site required for method reliability.	Able to tolerate pressure and immersion challenge. Rigid, or flexible with package restraint mechanism.	**Row 4** Varies with container–closure, test sample fixtures and positioning, challenge condition severity, and inherent biological variability.	Qualitative measure by visual inspection of microorganism growth inside test samples filled with growth-supportive media or product, post immersion in heavily contaminated challenge media while exposed to differential pressure conditions, followed by incubation to encourage microbial growth. Growth in the test sample indicates the presence of test sample leak site(s) capable of allowing passive or active entry of microbes.	Destructive	Weeks
Tracer gas detection, sniffer mode	Tracer gas must be added to package. Tracer gas must have access to package surfaces to be tested for leaks.	Leak site accessible to probe. Limited tracer gas permeability.	**Row 2** Varies with test sample, method parameters, test sample fixtures, and analyst technique and skill. Smaller leak detection may be possible under optimum test conditions.	Quantitative measure by spectroscopic analysis of tracer gas near the outer surfaces of the tracer-flooded test sample, sampled using a sniffer probe. Tracer presence above a pass/fail limit indicates leak presence and location.	Nondestructive, unless tracer gas introduction to the package interior compromises test sample barrier.	Seconds to minutes
Tracer liquid	Contents must be compatible with liquid tracer. Product must not clog leak path.	Rigid, or flexible with package restraint mechanism. Able to tolerate liquid immersion. Compatible with liquid tracer detection mode.	**Row 4** Varies with container–closure, test sample fixtures and positioning, challenge condition severity, and tracer liquid content. Smaller leak detection may be possible under optimal test conditions employing chemical analysis tracer detection.	Measure of tracer in test sample previously submerged in tracer-charged liquid while exposed to differential pressure conditions. Alternatively, tracer-charged test samples may be submerged in tracer-free collection fluid. Tracer migration measurement may be quantitative (by chemical analysis; preferred approach for small leak detection) or qualitative (by visual inspection). Tracer presence indicates leak site(s) capable of allowing tracer passage. Tracer magnitude may indicate relative leak size (assuming a single-leak pathway).	Destructive	Minutes to hours

[a] All methods apply to nonporous, rigid and flexible packages as per the scope of ⟨1207⟩.

[b] The leak detection limit cited for each technology refers to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Table 1* and is provided for information only. This information is intended to assist in early methodology selection. The validated leak determination limit for a product–package specific test method may deviate from these values.

2. DETERMINISTIC LEAK TEST TECHNOLOGIES

2.1 Electrical Conductivity and Capacitance (High-Voltage Leak Detection)

2.1.1 DESCRIPTION

The electrical conductivity and capacitance leak test (high-voltage leak detection, or HVLD) is an approach for detecting the presence, and potentially the location, of a leak(s) in the wall of a nonporous, rigid or flexible package containing liquid or semi-liquid product. Test analysis is based on quantitative electrical conductance measurements (*1–5*). HVLD leak tests are generally nondestructive to the package and to the product, although an evaluation of HVLD exposure impact on product physicochemical stability is advised.

The test is performed by first positioning the test sample (containing liquid product) onto an electrically grounded instrument test fixture. Alternatively, the test sample may be placed onto a transport system that will carry the test sample through an electrically grounded testing zone. Upon test start, an electrode uniquely designed for the product–package type under test exposes all or part of the test sample to a high-frequency, high-voltage, low-amperage current. The presence of a leak path in the proximity of an electrically conductive, liquid-formulation product results in a drop in the electrical resistance of the test

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sample, as shown by a spike in current passing through the test sample above a predetermined pass/fail limit established using negative controls.

2.1.2 APPLICATION

Rigid or flexible packages of nonporous components containing liquid or semi-liquid product may be tested:

- Package components must be relatively electrically nonconductive.
- Product must be electrically conductive, relative to the package.
- Product must not be flammable (i.e., not a combustion risk).
- Product must be near or at the leak inspection location at the time of the leak test.
- Solidified, electrically conductive product that blocks leak paths may be detected.
- Metal caps used to seal stoppered vial or cartridge packages conduct current, improving the likelihood of finding leaks under the cap.

HVLD tests are rapid, requiring no more than several seconds for a full scan of the test sample, thus making them appropriate for off-line testing, or as an on-line, 100% product inspection test method. This technology is useful for any product life cycle phase.

2.1.3 TEST EQUIPMENT

HVLD instrumentation comes equipped with tooling and/or a test sample transport system for proper test sample and probe/ground positioning, an internal high-voltage transformer, electrode voltage and ground potentiometer adjustment capabilities, and a test result output display. The design and materials of construction used for the electrode probe and electrical ground are product–package specific.

2.1.4 TEST PARAMETERS

The following are test parameters for the electrical conductivity and capacitance leak test, also known as HVLD:

- Conductivity of test sample product relative to test sample package: a greater difference will improve leak detection sensitivity
- Test voltage set point: voltage is set high enough to ensure leak detection, but not so high that current will arc, falsely rejecting the test sample
- Test sensitivity set point (potentiometer or gain set point): sensitivity should be maximized to ensure leak detection without triggering a false reject result
- Package content proximity to potential leak paths: leak detection sensitivity is directly related to the proximity of product to the leak path
- Electrode probe position relative to potential leak paths: probe proximity to the leak is directly related to the test method sensitivity
- Speed at which the electrode passes over the test sample surface: although the test is very rapid, test speeds too rapid may cause leaks to be missed
- Moisture presence on the package: test sample surface condensation can potentially trigger a false reject reading

2.2 Laser-Based Gas Headspace Analysis

2.2.1 DESCRIPTION

Gas headspace analysis via laser-based techniques provides a quantitative, nondestructive measure of oxygen content, water vapor content, and low internal pressure in the headspace of a nonporous, rigid or nonrigid package (*6–8*). Some instruments are capable of measuring headspace carbon dioxide concentration as well.

The test is performed by first placing the test sample in a fixture designed for precise test sample positioning. Upon test start, frequency-modulated spectroscopy is used to cause a near-infrared (IR) diode laser light to pass through the gas headspace region of the sealed test sample. Light is absorbed as a function of gas concentration and pressure. The absorption information is processed using phase-sensitive detection techniques; a mixer demodulates the signal. The output voltage, which is proportional to the absorption line shape, is digitally converted and further analyzed by a microprocessor, yielding test sample signal results. Final test sample readings are automatically generated based on a comparison of test sample signals to a calibration curve. This curve is pre-established by using control packages flooded with traceable gas reference standards. Gas headspace analysis, as a function of time, provides a quantitative measure of the total leakage rate of the test sample. Leakage rates are judged acceptable or unacceptable on the basis of predetermined limits, calculated to ensure proper gas headspace content maintenance over the product life cycle.

2.2.2 APPLICATION

Rigid or flexible packages made of nonporous components (transparent or semi-transparent material, either amber or colorless) that allow transmission of near-IR diode laser light may be tested. Test samples require a minimum headspace volume and headspace path length. The requirements vary on the basis of the gas moiety to be tested and may be specific to the instrument as well as to the construction and design of the package materials.

Test samples that may be analyzed fall into these categories:

- Products that require low-oxygen or low-carbon-dioxide headspace content

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- Products that require low water vapor content (e.g., lyophilized or powdered products)
- Products that require low internal package pressure (e.g., lyophilized products)

Package integrity, or absence of leakage, is confirmed by replicating tests on a given test sample as a function of time. Longer time periods between tests are needed to detect smaller leaks. Mathematical models appropriate to leak flow dynamics may be used to predict the time required for detecting leaks of various sizes or rates.

Headspace analysis at a single time point provides the headspace content result, which may or may not be indicative of package integrity.

- A test result not meeting specification could be due to package leakage, or could result from improper package filling or assembly processes that caused the package headspace to be out of specification.
- A test result that meets specification may confirm package integrity if enough time has elapsed since product–package preparation for measurable leakage to have occurred, assuming that the initial preparation of the test sample met manufacturing standards.

Methods of laser-based gas analysis may be used during any phase of the product life cycle. Tests are rapid and are appropriate for off-line testing using lab-scale equipment (typical measurement time, 2 s) or as an on-line, 100% product inspection method (typical measurement time, 0.2 s).

2.2.3 TEST EQUIPMENT

Test instrumentation for laser-based gas headspace analysis is capable of accurate and reproducible near-IR diode laser light emission, light detection, and signal analysis. Tooling specific to product–package test samples is used to properly position test samples, ensuring reproducible laser-light transmission and detection. Standards with components identical to the packages under test, in terms of both the materials of construction and the dimensions (at the point where light is to be transmitted), are required. These standards also need to contain headspace content that is representative of the gas mixture under test (i.e., oxygen, carbon dioxide, water vapor, pressure).

2.2.4 TEST PARAMETERS

The following are test parameters for laser-based gas headspace analysis:

- Test sample position with respect to laser beam transmission and detection points: imprecise test sample handling, and/or dimensional irregularity of the package, can increase measurement standard deviation
- Test sample headspace volume: the volume must meet minimum requirements for test instrumentation
- Test sample headspace absolute pressure: headspace absolute pressure will influence the detection limit and range for all test types
- Test sample speed: increase in testing speed will increase standard deviation of measurement
- Test sample temperature: temperature can influence moisture and pressure test results (e.g., lower temperatures are associated with lower internal pressure of the test sample) (*9*)
- Test sample outer surface moisture: presence of moisture may hamper test performance
- Time allotted between the replicate tests: performing replicate tests over a period of time allows calculation of continuous package leakage

2.3 Mass Extraction

2.3.1 DESCRIPTION

The mass extraction test is a nondestructive, quantitative measurement approach for detecting leakage in nonporous, rigid or flexible packages (*10*). Leakage of package headspace gases and/or leakage below the product fill level may be detected, given appropriately designed equipment and test parameters.

The test is performed by first placing the test sample inside a test chamber that is pneumatically connected to a mass extraction leak test system equipped with a vacuum generator package. The test chamber is uniquely designed to contain the test package, which is fitted with appropriate tooling to limit movement or expansion of moveable or flexible components, respectively.

Upon test start, the chamber is quickly evacuated for a predetermined time to reach a predetermined vacuum level. A series of such evacuation cycles may be performed, each intended to identify smaller leakage rates. After each cycle, the test system is isolated from the vacuum source and measurements of absolute pressure, pressure decay rate, and/or gas mass flow rate are captured. Readings greater than predetermined limits that were established using negative controls are indicative of container leakage, triggering test cycle abort.

For those test samples passing all previous larger leak vacuum cycles, a final vacuum is drawn. The test system is then isolated from the vacuum source. With all flow from the test chamber directed through the mass flow sensor, the mass flow rate is measured. Mass flow above a predetermined limit established using negative controls is indicative of container leakage.

2.3.2 APPLICATION

Nonporous, rigid or flexible packages may be tested. Packages containing gas, liquid, and/or solid materials can be tested:

- Flexible packages or packages with nonfixed components require tooling to restrict package expansion or movement, respectively, when exposed to test vacuum conditions. Tooling minimizes the seal stress of flexible packages and maintains consistent package volume and differential pressure conditions across the leak path.
- Gas headspace must be at atmospheric pressure or at a pressure notably greater than test vacuum conditions.

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- Package surfaces below the product-fill level may be leak tested for those solid-formulation products that do not block leak-site gas flow and for those liquid products that volatilize at test vacuum but do not solidify and so block leak paths.
- Packages ranging in volume from a few milliliters to several liters may be tested.

Tests require anywhere from several seconds to a few minutes to perform. Longer test times are necessary for testing larger-volume packages. Lengthening test cycles also allows for detection of smaller leaks.

Mass extraction leak tests are useful in any phase of the product life cycle. Tests may be performed in a laboratory setting or off-line in the production environment. Longer laboratory or off-line test cycle times are generally capable of detecting smaller leaks. Higher speed on-line tests are restricted to larger leak detection.

2.3.3 TEST EQUIPMENT

Mass extraction test instrumentation consists of a system of conduits and valves that pneumatically connect a test chamber with a test system pressure sensor, micro-flow mass sensor, and vacuum generator package, including an external vacuum source. The instrument includes appropriate timers, electronic controls, and monitors. A fixed-size orifice is included for periodic system performance verification. The test chamber is uniquely designed to contain the test package, which is fitted with appropriate tooling to limit movement or expansion of moveable or flexible components, respectively.

2.3.4 TEST PARAMETERS

The following are test parameters for mass extraction:

- Pressures
  - Test system pressure reading after initial gross leak check. At evacuation stage, pressure is a function of test system volume, time allotted for evacuation, and the vacuum source pressure level.
  - Test system pressure reading after the secondary evacuation stage(s): pressure above a predetermined limit is due to test package leakage. The pressure level above baseline is a function of leak size, available headspace volume, and/or volatile liquid in the test sample.
  - The final absolute pressure of the test cycle must be lower than the headspace pressure of the test package for detection of gas headspace leaks, and/or lower than the volatilization pressure of liquid product formulation for leaks located below the liquid-product fill level.
- Mass flow
  - Mass flow rate reading after secondary evacuation stage(s).
  - The mass extracted from the test sample is monitored for larger leak detection after the first secondary evacuation stage; mass extracted from the test sample is monitored for the smallest leak detection after the last evacuation stage.
  - Background flow level (i.e., the baseline flow or noise level) is the flow rate for packages without leaks. Baseline flow is a function of test package and system outgassing, test system volume, and the time allowed for evacuation.
  - The mass flow rate at steady-state conditions, when extracted from the test chamber, is equal to test sample leakage into the test chamber, assuming that outgassing from the sample and external leakage from the test system are insignificant. Leakage is identified once the mass flow rate notably exceeds the rate of negative controls.
- Times
  - Time allotted for system evacuation for gross leak detection: enough time is allotted to draw off most of the test chamber gases, without exhausting the headspace gases of the test package, or without drawing off the liquid contents from grossly leaking packages.
  - Time allotted for large-leak check through the mass flow sensor: a brief time is required for detection of large- and medium-sized defects.
  - Time allotted for system evacuation for small leak detection: enough time should be allowed to establish the desired vacuum equilibrium of the test chamber. Insufficient time will not adequately draw off gases sorbed onto package surfaces or entrapped between components.
  - Time allotted for mass flow to stabilize: after the secondary evacuation stage(s), monitor the flow as the flow rate approaches steady state. Enough time is allotted so that flow from the smallest allowed defect is statistically greater than baseline (no-leak) flow.
  - Time allotted for the final leak test by mass flow: enough time should be allotted so that the mass flow rate exceeds baseline readings for negative controls.

2.4 Pressure Decay

2.4.1 DESCRIPTION

The pressure decay test is a quantitative measurement approach for detecting leakage in nonporous, rigid or flexible packages. The test is destructive if the introduction of pressurized gas creates a break in the package wall or seal. The test is nondestructive if the introduction of gas into the test sample does not compromise the package barrier. Pressure decay testing is intended for integrity testing of the gas headspace region of the test sample.

To perform the test, a dry air or inert gas pressure source is attached to the test sample that is fitted with an internal pressure monitoring device. The test sample is pressurized to a predetermined pressure, after which the pressure source is isolated from the test sample. The decay in pressure is monitored for a predetermined time. Pressure decay that exceeds a predetermined limit established using negative controls indicates container leakage.

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The referenced ASTM F2095 method (*11*) is intended for testing flexible packages (pouches and foil-sealed trays). Seals or surfaces being tested cannot be in contact with product such as water, oils, or other liquids. ASTM method A describes testing packages without use of a restraint mechanism. The method requires that the package reach a stable volume configuration (i.e., it stops stretching) to take a measurement. ASTM method B requires that the test sample is kept between restraining plates during the test to limit the volume of the pressurized package.

2.4.2 APPLICATION

Nonporous, rigid or flexible packages may be tested:

- Package surfaces that can be tested are those unobstructed by product (e.g., oils, water, or other liquids); small leaks below the liquid-fill level would not be detected by this method.
- Flexible packages or packages with nonfixed components require tooling to restrict package expansion or movement, respectively, when exposed to test pressure conditions. Tooling minimizes the seal stress of the flexible package and maintains consistent package volume and differential pressure conditions across the leak path.
- Pressure decay can be used for testing packages anywhere from a few milliliters in volume to large, bulk-storage vessels.

Tests require anywhere from a few seconds to a several hours to perform. Longer test times are necessary for testing larger-volume containers. Lengthening test cycles also allows for detection of smaller leaks.

Pressure decay tests are useful in any phase of the product life cycle.

Tests may be performed in a laboratory setting or off-line in the production environment. Laboratory or off-line test equipment that allows for longer test times is generally capable of detecting smaller leaks. Higher-speed, on-line pressure decay equipment may be used to check for defects in open packages before package filling and closure.

2.4.3 TEST EQUIPMENT

Pressure decay test instrumentation includes conduits to connect the test sample with test system pressure transducers (absolute, differential, or a combination of both) and a pressure source (*12*). Instrumentation includes appropriate timers, electronic controls, and monitors. Greatest test method sensitivity and reproducibility are achieved when the instrument is kept in a temperature-controlled environment; test samples (especially larger-volume samples) are kept at a controlled, constant temperature during test; and dry pressurizing gas at constant temperature is used. It is optional to use tooling uniquely designed to limit movement or expansion of moveable or flexible components, respectively, thereby keeping test sample volume constant and limiting seal stress.

2.4.4 TEST PARAMETERS

The following are test parameters for pressure decay:

- Test sample internal pressure after pressurization:
  - The initial pressure reached after sample pressurization is a function of the test system volume, time allotted for pressurization, pressure source capacity, and temperature of the test sample headspace.
  - Higher pressure creates the potential for more rapid and sensitive leak testing. However, the selection of maximum test pressure should take into consideration personnel safety risks and potential damage to equipment and package.
- Pressure decay baseline: the baseline pressure decay (i.e., noise level) is the pressure drop that occurs for packages without leaks.
  - Baseline pressure drop is a function of test package volume, temperature conditions, and the length of time allowed for pressure to rise.
  - Baseline pressure drop requiring longer time periods is affected by gas sorption onto test package surfaces, gas moisture content (dry gas should be used), and gas temperature. Techniques to limit baseline pressure drop include the use of dry gases, and keeping the test container and the pressurized gas at a constant temperature.
- Pressure decay due to test package leakage: the extent of pressure decay above baseline is a function of leak size, available headspace volume in the test sample, the initial pressure inside the test sample, temperature control, and the time allotted for pressure to rise.
- Times
  - Time allotted for test sample pressurization: enough time is allotted to establish the desired pressure inside the test sample.
  - Time allotted after pressurization for pressure decay: enough time should be allotted so that the pressure decay from the smallest leaks can be detected (i.e., baseline decay is exceeded).
  - A time lag may be incorporated before monitoring for pressure decay to allow for gas equilibrium within the container and test system.
- Temperatures
  - Temperature of the pressurized gas can significantly affect test method sensitivity and reliability, especially when testing larger-volume containers.
  - An increase in gas temperature causes a rise in pressure, and conversely, a rise in gas pressure triggers an increase in gas temperature. Therefore, upon initial test package pressurization, the gas temperature will spike, causing a further spike in pressure. The subsequent drops in temperature and pressure during system equilibrium may be mistaken for leakage.

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- Effects of temperature variation may be minimized by: 1) allowing the temperature in the pressurized system to come to equilibrium before starting the pressure decay test, 2) minimizing temperature variation outside the test system, 3) minimizing test duration, and/or 4) applying a mathematical correction to the pressure readings.

2.5 Tracer Gas Detection, Vacuum Mode

2.5.1 DESCRIPTION

The leak detection method for tracer gas detects leakage from nonporous, rigid or flexible packages. The method can be destructive or nondestructive, depending on the test approach used. The test requires the presence of tracer gas inside the test sample package. Helium is the most commonly used tracer gas, and hydrogen is also used. The leakage rate of tracer gas is quantitatively measured using a spectrometric analytical instrument specific for the tracer gas. Instruments are designed to check for tracer gas leaking out of a test package, either by means of a test chamber that can be evacuated to draw gas out of test sample leaks (the vacuum mode) or by use of a vacuum wand (the sniffer mode) for scanning the outer surfaces of the test package (see *Tracer Gas Detection, Sniffer Mode*). The vacuum-mode tracer gas test is used more commonly than the sniffer mode for integrity testing sterile pharmaceutical product–packages. The vacuum mode is both quantitative and deterministic and is used to capture and quantify leakage from an entire test package, or it can be used to test for leakage along a test package surface or seal, given proper sample fixtures. Both vacuum and sniffer testing modes using helium as the tracer gas are described in ASTM F2391 (*13*).

To perform the vacuum mode test, test samples that have been fully or partially flooded with tracer gas are placed inside an evacuation chamber that is pneumatically connected to the tracer gas analysis instrument. Alternatively, the test sample may be fitted in such a manner that only the surface or seal of interest is exposed to the instrument, allowing for targeted leak detection at one specific seal or surface. In some cases, test samples that cannot withstand the high-vacuum test conditions may be tested with the use of tooling to restrict package expansion or movement.

At test start, the instrument's vacuum pump evacuates the test chamber or fixture, drawing leaking tracer gas through the analyzer. The absolute leak rate of the test sample is calculated by normalizing test results by the partial pressure of the tracer gas within the test sample at the time of test. For accurate results, tracer gas concentration within the sample must be uniform and consistent at the time of test; also, there should be minimal tracer gas permeation out of the test sample that can mask test sample leakage. Calibration tracer gas reference standards can be used for understanding the relationship between true leak rates and measured leak rates under actual test conditions.

The vacuum-mode tracer gas leak test is a nondestructive test, unless tracer gas introduction into the test sample requires package wall compromise (e.g., piercing), or if the presence of tracer gas is detrimental to the package contents.

2.5.2 APPLICATION

Rigid or flexible packages made of nonporous components:

- Flexible packages or packages with nonfixed components may require tooling to restrict package expansion or movement, respectively.
- Tracer gas permeation through the package material must not be so great that the leakage rate of concern is masked.
- A wide range of package sizes may be tested.

Leak paths must be clear of liquid or solid materials that could potentially block tracer gas flow.

Caution is advised when testing liquid-filled packages, because vapors or liquid drawn into the test system can seriously damage instrumentation.

Detection capabilities range from large leaks to the smallest leaks.

- Method capability is related to the size of the unobstructed leak path.
- Large leaks in the smallest packages may be missed because of the rapid loss of tracer gas (e.g., during the evacuation phase of the vacuum mode test).
- Significant tracer gas permeation through the package itself can interfere with the test by swamping leakage rate.

The test is nondestructive if the tracer gas is introduced into the package at the time of package assembly or closure, but the inclusion of tracer gas may prevent introduction of these packages into commercial or clinical markets. The test is destructive if the introduction of tracer gas compromises assembled package integrity (e.g., package puncture). Following test sample preparation, the actual leak test generally takes less than 1 min.

Tracer gas leak test methods may find application in any product life cycle phase. They are generally used in a laboratory environment. Tracer gas methods can also be used in production as an off-line testing approach; they can be used on-line if tracer gas is introduced into the test samples before final package closure.

2.5.3 TEST EQUIPMENT

Analytical instrumentation specific for tracer gas detection (e.g., mass spectrometer for helium detection) is required, typically equipped with an internal leak rate standard for instrument calibration at time of use. Additional equipment needed includes external calibration reference standards of tracer gas, to be used for comparisons of true leak rate versus measured leak rate under actual test conditions; a tracer gas source with a means for introducing tracer gas into the test sample; a test chamber or fixture to pneumatically connect the test sample with the instrument; tooling to restrict package expansion or movement, as appropriate; a means for accessing and analyzing the test sample headspace for tracer gas partial pressure after execution of the vacuum-mode leak test; and ventilation to remove tracer gas from the test area, or a tracer gas recapture system.

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2.5.4 TEST PARAMETERS

The following are test parameters for tracer gas detection, vacuum mode:

- Tracer gas partial pressure within the test sample at the time of test:
  - When added by soaking the intact package, the concentration of the tracer gas is dependent on the time allotted, the positive pressure applied, and the tracer gas leak rate, plus the rate of permeation into the package
  - When added before package closure, tracer gas concentration is dependent on the gas flooding rate and time, the tooling and/or enclosure used to concentrate gas inside the package, and the efficiency of package closure for preventing escape of tracer gas
  - When added after package assembly, tracer gas concentration is dependent on the tooling used to pierce, flush, and vent the package; the gas flooding rate and time; and the sealant material applied to reseal the puncture site
- Differential pressure applied to the test sample during the vacuum mode will drive tracer gas out of the test sample, increasing method sensitivity
- Time allotted to allow for leakage to reach steady state should not exceed tracer gas permeation lag time

2.6 Vacuum Decay

2.6.1 DESCRIPTION

The vacuum decay test is a nondestructive, quantitative measurement approach for detecting leakage in nonporous, rigid or flexible packages. Leakage in the package headspace gas region and/or below the product-fill level may be detected given appropriately designed test parameters and if product properties allow (as detailed below).

To perform the test, the test sample is placed in a closely fitting evacuation test chamber pneumatically connected to the leak test system, which is equipped with an external vacuum source. The test chamber is uniquely designed to contain the test package. Test samples with moveable or flexible components require appropriate tooling to limit the movement or expansion of such components, respectively.

Upon test start, the test chamber plus test system dead space are evacuated for a predetermined period of time. The targeted vacuum level chosen for the test is predetermined on the basis of the test sample type, size, and content. The vacuum source is then isolated from the test system. After a short time has elapsed to allow for system equilibration, the rise in dead space pressure (i.e., vacuum decay) is monitored for a predetermined length of time using absolute and/or differential pressure transducers. A pressure increase that exceeds a predetermined pass/fail limit established using negative controls indicates container leakage. ASTM F2338 may be referenced (*14*).

2.6.2 APPLICATION

Nonporous, rigid or flexible packages may be tested. Packages containing gas, liquid, and/or solid materials can be tested:

- Flexible packages or packages with nonfixed components require tooling to restrict package expansion or movement, respectively, when exposed to test vacuum conditions. Tooling minimizes flexible package seal stress and maintains consistent package volume and differential pressure conditions across the leak path.
- Product–package gas headspace must be at atmospheric pressure or at a pressure notably greater than test vacuum conditions.
- Package surfaces below the product-fill level may be tested for leaks for those solid dosage formulation products that do not block leak-site gas flow or for those liquid dosage form products that volatilize at test vacuum without solidifying and blocking leak paths.
- Packages ranging in volume from a few milliliters to several liters may be tested.

Vacuum-decay leak tests are useful in all phases of the product life cycle. Tests require anywhere from a few seconds to a few minutes to perform. Longer test times are necessary for testing larger-volume packages or for detection of the smallest leaks. Longer test times are more appropriately performed in a laboratory setting or off-line in the production environment. Higher-speed on-line equipment is generally used for detecting larger leaks.

2.6.3 TEST EQUIPMENT

Vacuum-decay leak test instrumentation consists of a system of conduits and valves that pneumatically connect a test chamber with the test system pressure sensors and an external vacuum source. The instrument includes appropriate timers, electronic controls, and monitors. An external gas flow meter allows for periodic system performance verification. The test chamber is uniquely designed to closely contain the test package and may be fitted with tooling to limit movement or expansion of moveable or flexible package components, as appropriate.

2.6.4 TEST PARAMETERS

The following are test parameters for vacuum decay:

- Pressures
  - Test chamber pressure after evacuation: the initial pressure reached during test chamber evacuation is a function of test system volume, time allotted for evacuation, and vacuum pump capacity. The absolute pressure of the test system must be lower than the headspace pressure of the test package for detection of gas headspace leaks, and/or lower than the volatilization pressure of the liquid-product formulation for leaks located below the liquid-product fill level.

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- Pressure rise baseline: the baseline pressure rise (i.e., noise level) is the pressure increase that occurs for packages without leaks. Baseline pressure rise is a function of test package and system outgassing, test system volume, and the time allowed for pressure rise.
- Pressure rise due to test package leakage: the extent of pressure rise above baseline is a function of leak size, test chamber vacuum level at test start, available headspace volume or volatile liquid in the test sample, and the time allotted for pressure rise.

- Times
  - Time allotted for system evacuation: enough time should be allowed for establishing the desired vacuum pressure level of the test chamber, plus draw off gases sorbed onto package surfaces or entrapped between components. Excessive time will evacuate headspace from largely leaking packages, risking that there will be insufficient headspace gas for leak detection. Times should not be so great that leaking package headspace gases are exhausted or the liquid product floods and contaminates the test system.
  - Time allotted after evacuation for pressure rise (vacuum decay): enough time should be allotted so that the pressure rise from the smallest leaks to be detected exceeds baseline. A time lag may be incorporated after evacuation and before vacuum decay monitoring to allow for gas equilibrium within the container and test system.

3. PROBABILISTIC LEAK TEST TECHNOLOGIES

3.1 Bubble Emission

3.1.1 DESCRIPTION

The bubble emission leak test is a destructive, qualitative measurement approach for detecting and locating leaks in nonporous, rigid or flexible packages containing headspace gas.

The test is performed in one of two ways. The first is an internal pressurization method referenced in ASTM F2096 (*15*) in which a positive pressure air source with pressure monitor is inserted into the test sample. The test sample is then submerged in water, and air pressure is applied to a predetermined level, for a predetermined time period. The second approach is referenced in ASTM D3078 (*16*). The intact test sample is submerged in water or other suitable submersion fluid contained in a vacuum chamber. Vacuum is established to a predetermined level, for a predetermined time period.

With both approaches, leakage can be observed as a continuous stream of bubbles emitted from the leak site. Bubble diameter and emission rate may provide some indication of relative leak size. An alternative to test sample submersion is coating the test sample with surfactant, in which case any leakage is seen as foaming or bubbling at the leak site. The surface tension of the submersion fluid or surfactant allows for smaller bubble formation, potentially improving test sensitivity. Use of submersion fluid with low gas solubility may also improve test sensitivity.

The bubble emission test is categorized as a probabilistic leak test method. Although this method relies on the predictable flow of gas through leak paths, escaping gas can become entrapped within or between package components; false-leak outgassing events may occur; gas emitted from small leaks may solubilize in the immersion fluid before bubble formation; and test sample set up may be inadequate to ensure sufficient differential pressure conditions and appropriate bubble visibility. The use of negative and positive controls along with test samples provides evidence of test method limit of detection.

3.1.2 APPLICATION

Nonporous, rigid or flexible packages with gas headspace may be tested by bubble leak methods:

- Packages must be able to tolerate wetting or submersion
- Flexible packages or packages with nonfixed components generally require tooling to restrict package expansion or movement, respectively, when exposed to vacuum conditions. Tooling minimizes the seal stress of the flexible package and maintains consistent package volume and differential pressure conditions across the leak path. However, tooling may block leak paths or hinder bubble emission visibility
- Only leak sites that are present in the gas headspace region of the package can be detected
- This test is generally used for testing smaller-volume packages that are less than a few liters in size

Bubble tests are applicable in any product life cycle phase. Bubble tests require several minutes or longer for test sample analysis and subsequent cleaning and/or drying. Bubble tests are most commonly used in laboratory settings as part of a research investigation to verify leak presence and location. They can also be used as an off-line production leak test. Bubble tests are also used for integrity testing of aerosol–package products in a research or production setting. In this application, the test may be considered nondestructive to the product–package test sample.

3.1.3 TEST EQUIPMENT

Bubble tests require a pressure or vacuum source (as appropriate) equipped with pressure monitors and controls; tooling to restrict expansion of flexible packages or movement of nonfixed components; submersion fluid or surfactant to be applied to the package surface; submersion vessel equipped for external vacuum or internal pressure test mode; and visual inspection aids such as lighting, magnification, and/or background, as needed.

3.1.4 TEST PARAMETERS

The following are test parameters for bubble emission:

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- Differential pressure: greater differential pressure evokes more rapid bubble emission. Differential pressure should not be so great that package seals are compromised or that gas escapes through large leaks so rapidly that it may be confused with package surface outgassing
- Time allotted during differential pressure application: longer test times allow for smaller leak detection
- Times allotted for inspection (pacing) and for inspection breaks to lessen operator fatigue
- Package positioning during inspection
- Package mode of restraint for moveable or flexible components
- Submersion fluid (or surfactant) surface tension: lower surface tension improves method sensitivity
- Inspection environment parameters: lighting intensity and angle, degree of magnification, background color

3.2 Microbial Challenge, Immersion Exposure

3.2.1 DESCRIPTION

The immersion exposure microbial challenge is a destructive, qualitative measurement approach for confirming leaks in nonporous, rigid or flexible packages.

The test is performed by first filling test samples with sterile, growth-supporting media, followed by incubation and visual inspection of samples to ensure sample sterility before microbial challenge. Samples are then immersed in a concentrated bacterial suspension for a predetermined time. Samples can be exposed during immersion to a predetermined vacuum for a predetermined time, followed by release of vacuum while the packages remain immersed at ambient pressure for a predetermined time. Samples are then incubated under growth-promoting conditions, followed by examination of package contents for evidence of microbial growth by visual inspection or other appropriate analytical means. Alternative approaches can include exposure of immersed test samples to positive pressure conditions, or to multiple cycles of vacuum and/or pressure conditions. Test sample leakage is evidenced by visible growth of the challenge microorganism(s) inside test samples. Immersion microbial challenge tests rely on the presence of a liquid carrier in the leak path that sweeps microorganisms into the package or provides a means whereby microorganisms can actively migrate and/or grow into the test sample. The use of negative and positive controls along with test samples provides evidence of test method limit of detection.

The microbial challenge by immersion test is categorized as a probabilistic leak test because of the multiple events that must occur sequentially and/or simultaneously for leak detection to take place. All such events are difficult to predict or control, especially for detection of smaller leaks. For example, the microorganisms must be physically present at the leak site. The necessary presence of liquid in the leak path, and/or flowing through the leak path, is influenced by the package materials of construction, leak path tortuosity and topography, media surface tension, and leak path blockage by product, extraneous debris, or air locks. Microorganisms must not be hindered from entering the package by getting trapped in a tortuous leak path, and enough microorganisms must enter the package to allow for sufficient growth that can be detected visually after test sample incubation.

3.2.2 APPLICATION

Microbial challenge tests by immersion are most useful when an appropriate and validated physicochemical leak test method does not exist, or when the test outcome demands direct evidence of the prevention of microbial entry.

Nonporous packages of rigid or flexible components may be tested by immersion microbial challenge methods:

- Packages must be able to tolerate submersion.
- Flexible packages or packages with nonfixed components may require tooling to restrict package expansion or movement, respectively. Tooling minimizes stress on flexible package seals and maintains consistent differential pressure conditions across the package seal.

Immersion microbial challenge tests are performed in a laboratory environment, not as an on-line test of the finished product.

A test requires several days to prepare; the test samples are pre-incubated before microbial challenge to ensure initial package content sterility. The challenge itself, plus post-challenge sample decontamination, may take several hours. Final incubation followed by sample inspection may take 1–2 weeks. The immersion microbial challenge test is primarily used in product–package development and validation studies.

3.2.3 TEST EQUIPMENT

The test chamber for conducting the immersion microbial challenge test is designed and equipped to maintain suspension uniformity and appropriate temperature while exerting the required differential pressure condition. Fixtures for restraining and/or positioning packages during immersion are also required. Small, motile microorganisms are preferred for the challenge; examples include *Brevundimonas diminuta* and *Serratia marcescens*. The immersion challenge media should support challenge microorganism growth to the desired concentration. Soybean-casein digest medium is one commonly used medium.

The media filled into test samples may match the immersion challenge media formulation. Alternatively, product that has been shown to support microbial growth may be used. Media should allow sufficient growth of the particular challenge organism so that package contamination can be detected in the positive controls. Verification of growth promotion in the immersion media and the test sample media should be performed each time the immersion challenge test is conducted (see *Sterility Tests* ⟨71⟩, *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*). Use of an incubation chamber is required for test samples, both before and after exposure. A means for determining microbial growth inside test packages is required.

3.2.4 TEST PARAMETERS

The following are test parameters for microbial challenge, immersion exposure:

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- Microorganism concentration: in the immersion challenge media, microorganism concentration should meet appropriate predetermined levels throughout the challenge test (a minimum concentration of 10^5 CFU/mL is commonly used).
- Media fill volume: the fill volume of media in test samples should be sufficient to ensure a liquid path at each potential leak site. Exposure of package seals and leak sites to the media may also be accomplished by test sample positioning during the immersion challenge. It is also necessary to have sufficient volume and correct composition of the package headspace to encourage growth of the selected challenge microorganism. Note that when the composition of the package headspace does not include oxygen, other test conditions (e.g., anaerobic) may be applicable.
- Vacuum/pressure conditions: exposing immersed test samples to differential pressure conditions is an important method parameter that serves multiple purposes:
  - Differential pressure helps eliminate trapped air and ensures the presence of liquid media between package components and at leak sites.
  - Differential pressure simulates the pressure changes incurred during air or land freight transport of the product. Absolute pressure conditions that correspond to various altitudes anticipated during land and/or air freight transport are provided in ASTM D6653/D6653M (*17*). Differential pressure conditions of the test may be modified on the basis of knowledge of the product–package shipping environment.
  - Differential pressure exposure can simulate conditions experienced by the product during some sterilization treatments.
- Test times: longer exposure times improve the likelihood of microbial ingress into defective test samples and positive controls. However, a possible decrease in the ability of media to support growth over time must be considered.
  - Time allotted for immersion exposure during differential pressure exposure (vacuum and/or pressure)
  - Time allotted for immersion exposure at ambient pressure conditions
- Temperature during challenge: temperatures sufficient to support microbial growth are recommended. Temperature cycling can also be used as a tactic to eliminate airlocks and promote the presence of liquid media at package seal sites.
- Pre- and post-challenge test incubation temperature and times: the temperature selected should allow sufficient microbial growth. Incubation times should be sufficient to ensure visualization of growth; these times are determined on the basis of positive controls and samples from growth-promotion studies.
- Parameters for detecting post-incubation microbial growth (e.g., lighting and background color for visual inspection, handling procedure, and pacing).

3.3 Tracer Gas Detection, Sniffer Mode

3.3.1 DESCRIPTION

The following information is specific to the tracer gas detection performed in the sniffer mode. (For additional information, see *Tracer Gas Detection, Vacuum Mode*.)

Tracer gas detection using a sniffer attachment is used to detect leak presence and location in nonporous, rigid or flexible packages. This is a nondestructive leak test, unless tracer gas introduction into the test sample requires package wall compromise (e.g., piercing) or if tracer gas presence is detrimental to package contents. The sniffer mode test using helium as the tracer gas is described in ASTM F2391, Procedure A (*13*). Briefly, test samples are flooded completely or partially with the tracer gas via one of several options. These options may include piercing a closed test sample to introduce pressurized tracer gas (sealant is applied to close the puncture site); flooding the test sample before package closure; or "soaking" a closed test sample by pressurizing with tracer gas (most applicable to larger leak detection). Test samples are checked for leakage by scanning the outer package surfaces using a vacuum wand that is pneumatically connected to the tracer gas analytical test instrument (e.g., a mass spectrometer for helium detection). Calibration reference standards of tracer gas can be used for understanding the relationship between true leak rates and measured leak rates under actual test conditions. The use of negative and positive controls along with the test samples provides evidence of test method limit of detection.

The sniffer mode of tracer gas leak testing is a probabilistic leak test method. This is because the presence of concentrated tracer gas near the test sample surface is not a well-defined or predictable event, and the sniffer scanning procedure is prone to variability related to human technique. The sniffer mode is generally chosen when the leak location is to be identified.

3.3.2 APPLICATION

Rigid or flexible packages made of nonporous components may be tested:

- Flexible packages or packages with nonfixed components may require tooling or manual manipulation to force tracer gas through leak paths.
- Tracer gas permeation through the package material must not be so great that the leakage rate of concern is masked.
- A wide range of package sizes may be tested, ranging from small packages to large multi-liter vessels.

Leak paths must be clear of liquid or solid materials that could potentially block tracer gas flow. The leak size detection capability is related to an unobstructed leak path.

The sniffer probe must not be allowed to draw liquid or hazardous vapors into the test system, as this would risk serious instrument damage.

Tracer gas leak test methods require time for the introduction of tracer gas into the test package, and up to several minutes to scan the package. Large leaks in the smallest packages may be missed because of the rapid loss of tracer gas.

Methods of tracer gas leak tests in the sniffer mode are generally used in the laboratory environment for locating package leaks. Tracer-gas sniffer mode tests are useful in any product life cycle phase.

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3.3.3 TEST EQUIPMENT

Test equipment for tracer gas leak detection is described in *Tracer Gas Detection, Vacuum Mode*. However, a sniffer probe with an enclosure and/or tooling for concentrating tracer gas passing through smaller leaks is used, rather than the test chamber or test fixtures described in *Tracer Gas Detection, Vacuum Mode*.

3.3.4 TEST PARAMETERS

The following are test parameters for tracer gas detection, sniffer mode:

- Differential pressure applied to the test sample will drive tracer gas out of the test sample, increasing the method's sensitivity. For flexible packages, differential pressure can be exerted by package compression.
- Tracer gas partial pressure within the package at the time of the test is discussed, with instructions, in the *Tracer Gas Detection, Vacuum Mode*.
- Aspects of the sniffer mode vacuum wand, such as sweeping speed and distance from the package surface, as well as the tooling or enclosure used for concentrating leaking gas.

3.4 Tracer Liquid

3.4.1 DESCRIPTION

The tracer liquid test method is a destructive approach for detecting and potentially locating leaks in nonporous, rigid or flexible packages. Tracer liquid tests provide an indication of leak presence and may provide a measure of relative leak size. Tracer liquid tests using liquid submersion work by the diffusive flow of the tracer element through a liquid-filled leak path and/or the effusive flow of tracer solution through the leak path.

The liquid submersion test uses one of two basic approaches. In the first approach, test samples are submerged in a tracer-element solution formulation contained in an evacuation chamber. Examples of tracer elements include dyes, radionuclides, or metallic ions. In the second approach, test samples containing tracer formulation are submerged in tracer-free liquid contained in an evacuation chamber. For both approaches, test samples may be fitted with tooling to ensure proper positioning and to restrict flexible or moveable components. The submerged test samples are subjected to vacuum at a predetermined pressure level for a predetermined time. After vacuum release, test samples remain submerged for a predetermined time. Additional test options include the use of positive pressure exposure or multiple cycles of differential pressure conditions to encourage effusive flow of the tracer element through the leak path.

In the first approach, after the challenge is complete, test sample outer surfaces are cleaned, and the contents are checked for evidence of tracer ingress (*18*). In the second approach, after the challenge is complete, the immersion fluid is checked for evidence of tracer liquid egress out of the test sample. In both cases, measurement of tracer liquid migration may be performed in a quantitative manner by using chemical analysis techniques (preferred for small leak detection). Alternatively, the presence of leakage may be determined qualitatively by visual inspection if the tracer element can be discerned visually. The use of negative and positive controls along with test samples provides evidence of test method limit of detection.

Liquid tracer tests are categorized as probabilistic methods. Successful liquid tracer detection relies on a combination of tracer solution wicking, tracer solution effusion, and tracer element diffusion through a liquid-filled leak path and are events that are difficult to predict or control, especially for detection of smaller leaks. These events are influenced by numerous factors, including the package materials of construction, leak path tortuosity and topography, tracer liquid surface tension, and leak path blockage by product, extraneous debris, and air locks.

3.4.2 APPLICATION

Rigid or flexible packages of nonporous components may be tested using tracer liquid submersion methods:

- Packages must be able to tolerate wetting or submersion.
- Flexible packages or packages with nonfixed components may require tooling to restrict package expansion or movement, respectively.
    - Testing by submerging a test sample in tracer liquid is used when the test sample allows for visual examination of the tracer (e.g., dye) ingress, or when the tracer element is to be contained within the test sample after testing.
    - Testing by submerging tracer-filled test samples in tracer-free liquid may be used when the sample interior cannot be visually examined or when the tracer element is best captured for analysis outside the test sample.

Tests may require up to 1 h or longer for test sample exposure, cleaning, and inspection or analysis. Tracer liquid tests are primarily used for laboratory testing or off-line product sample testing. Tracer liquid submersion tests can be used in any product life cycle phase.

3.4.3 TEST EQUIPMENT

A test vessel equipped for challenge conditions of vacuum and/or positive pressure is required and is pneumatically connected to a pressure and/or vacuum source, as appropriate, and equipped with pressure monitors and controls (*19*). The tracer liquid formulation should be physicochemically compatible with the test sample components and the tracer-free solution to be filled into the test samples or used as the immersion bath, to ensure optimal tracer solution functionality. Formulation considerations include tracer element type and concentration; surfactant use, type, and concentration; and solvent system. Examples of tracer liquid incompatibilities apparent upon contact with the test product include tracer dye fading, tracer element precipitation, and tracer element sorption onto package components. Tooling is often needed to restrict flexible package expansion or

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nonfixed component movement upon differential pressure exposure. Tooling minimizes stress on flexible package seals and ensures consistent differential pressure conditions across the leak path.

Tracer detection requires the use of either analytical detection instrumentation [e.g., UV-Vis spectrophotometry (*20*), phase induction spectrophotometry, or other] or visual inspection aids. Analytical detection offers the advantage of minimizing the error that is inherent in visual discernment of low dye concentrations. Optimal visual inspection requires the use of controlled inspection conditions, such as background color, lighting, pacing, fatigue breaks, and negative controls for comparison.

3.4.4 TEST PARAMETERS

The following are test parameters for tracer liquid:

- Differential pressure conditions used (vacuum and/or pressure): greater pressure differentials encourages tracer liquid passage.
- Submersion times during and after differential pressure application: longer times allow for greater tracer liquid passage through leak paths.
- Holding time between tracer liquid challenge and final inspection: some tracer liquids visibly fade or are sorbed onto package surfaces over time.
- Tracer liquid surface tension: lower surface tension allows for smaller leak detection.
- Tracer detection parameters
  - Analytical detection
    - Test sample content extraction procedure
    - Method-specific performance parameters
  - Visual inspection
    - Inspection environment parameters: lighting intensity and wavelength, background color, viewing angle, and test sample visibility
    - Time allowed for inspection (pacing) and breaks to lessen operator fatigue
    - Comparison with negative controls

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⟨1207.3⟩ PACKAGE SEAL QUALITY TEST TECHNOLOGIES

1. INTRODUCTION
2. CLOSURE APPLICATION AND REMOVAL TORQUE
3. PACKAGE BURST
4. PACKAGE SEAL STRENGTH
5. RESIDUAL SEAL FORCE
6. AIRBORNE ULTRASOUND

1. INTRODUCTION

The purpose of this chapter is to briefly summarize test methods useful for characterizing and monitoring package seal quality and to guide the reader in their selection and use. These methods are not leak tests but provide additional data regarding package seal characteristics that may affect package integrity and leakage.

"Package seal quality tests" are checks used to characterize and monitor the quality and consistency of a parameter related to the package seal, providing some assurance of the package's ability to maintain integrity. Seal quality tests ensure that seal attributes, package materials, package components, and/or the assembly process are consistently kept within established limits, thus further supporting package integrity. Seal quality tests differ from leak tests in that they provide no information relative to actual package integrity; thus, a package that meets the requirements of a seal quality test may still be defective and leak. For example, a flexible pouch package that passes a seal strength test may leak through a puncture in the pouch face. A bottle that meets closure application and removal torque tests may have a scratch on the bottle finish surface that allows product leakage. In contrast, a pouch or bottle that is poorly assembled could pass leak tests at the time of product manufacture, yet develop leaks later, before reaching the end user.

Therefore, seal quality tests and leak tests work together to ensure package integrity. The package seal quality tests described in this chapter were selected for inclusion on the basis of data in peer-reviewed scientific publications and/or data regarding recognized standard tests (e.g., precision and bias study results). Standard test methods (e.g., ASTM) are referenced where applicable (the reader is advised to utilize the most recent versions). In some cases, the scope of referenced standard test methods does not include the package types of the scope in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩. All methods and literature references are cited to provide benchmark information useful for the application and use of pharmaceutical package seal quality test methods.

Unlike package leak tests, seal quality tests are qualified for use rather than being fully validated. Qualification includes a demonstration of instrument performance and in some cases proof of appropriate instrument set-up specific for the package to be tested.

Finally, this chapter is not intended to provide an exhaustive listing of all seal quality technologies that could be used. Nor is the use of a methodology cited meant to be compulsory. Other qualified tests that are not included in this chapter may be used, as appropriate.

2. CLOSURE APPLICATION AND REMOVAL TORQUE

The closure application torque test measures the force exerted during the application of a screw-thread cap onto a threaded container. Conversely, the closure removal torque test measures the force required to initiate screw-cap removal. The container–closure systems of some ophthalmic solution products are examples of sterile product packaging closed with screw-thread caps.

Cap application torque is kept within an optimum range to prevent leakage from loose caps and to preclude component distortion and compromised seals from over-torqued caps. Cap removal torque is less than application torque due to stress relaxation and closure "back off" that may occur as a function of time and other environmental variables. A properly designed and applied cap will retain sufficient sealing force until the package is opened at the time of use. Caps that back off excessively during shipping, storage, or distribution increase the risk of product leakage. For multiple-dose packages, cap design and application forces should be such that the end-user population may be able to open and reclose the package in a manner that properly preserves the pharmaceutical product.

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Reproducibility of application/removal torque test results is improved when tests are performed using automatic instrumentation that applies and removes caps at uniform speeds, with fixtures to lock the bottle and cap into proper test position. Application and removal torque tests are described in several standard test methods that have been written to support various continuous-thread and child-resistant cap designs, including the ASTM methods referenced (*1–7*).

3. PACKAGE BURST

The package burst test is performed by inserting a pressure source into a test package and applying pressure until the package seal(s) burst open. Packages having seals that could be compromised when exposed to a net positive pressure force inside the package may be tested by this method. Such package types include flexible bags and pouches formed by heat-sealing processes.

The package burst strength test result (reported in pressure units) provides an indication of relative seal strength and evaluates the most likely location and mode of package failure when the package is exposed to a pressure differential. However, note that this test method cannot provide a measure of package seal uniformity or overall package integrity.

Package burst tests are described in ASTM F2054 (*8*) and ASTM F1140 (*9*) (Test method A). Method ASTM F2054 uses a restraining plate to limit package expansion during inflation. A restraining plate ensures that the stress is uniformly applied to all seal areas, allowing for identification of the weakest point along the seal. Additional factors can influence the test results, including package inflation rate, tooling dimensions (including gap height), and pressure-sensing mechanisms.

Method ASTM F1140 uses no restraining plate; therefore, this approach provides a snapshot of package performance when challenged with differential pressures in a typical sterilization cycle or distribution environment, for example. However, without restraining plates, stress applied to the package is highest at the middle of the package where the package inflates to the greatest diameter; therefore, the weakest area of the seal may not be identified by this approach. Package inflation rate, as well as pressure-sensing mechanisms and their detection limits, can influence test results.

4. PACKAGE SEAL STRENGTH

The package seal strength test, commonly known as the peel test, measures the force required to peel apart two bonded surfaces. This test is applicable for testing seals between two bonded, flexible surfaces (e.g., a pouch or bag) or between a flexible material and a rigid material (e.g., a lidded tray). Force results provide a measure of seal strength between the bonded surfaces. Maximum seal force, as well as average force to open the seal, may be determined with this method. The results are also useful for monitoring the consistency of package assembly.

The test is performed using a universal stress-strain instrument set to extension force mode, with special tooling for correctly positioning and holding the test sample. The seal strength test is described in ASTM F88 (*10*). Test results are affected by the peel test fixture design, the peel angle, the pull direction, pull speed, and properties of the test sample itself.

5. RESIDUAL SEAL FORCE

The residual seal force (RSF) test provides an indirect measure of the compressive force exerted by an elastomeric closure onto a parenteral vial finish after package assembly (capping). RSF tests can be performed on glass or plastic vial packages of all dimensions. A consistent and sufficiently significant RSF value provides a useful indicator of capping process consistency. RSF is linearly related to closure compression: more tightly capped vials yield higher RSF values.

The RSF test is performed using a universal stress-strain instrument, set to compression force mode, and a metal tool (called a cap anvil) designed to fit on top of the sealed vial package. A constant rate of compressive strain is exerted onto the anvil, which rests on top of the vial package, yielding a typical plot of stress as a function of time (or compressive distance). The point in the stress-response curve immediately before the terminal slope change corresponds to the capped closure's RSF, reported in either newtons or pound-force units. RSF tests are nondestructive in terms of package integrity. Test results are influenced by cap anvil tooling design, test compression rate, closure viscoelastic properties, closure dimensions, and the inclusion of an aluminum seal plastic top (more reproducible results are possible if plastic tops are removed before testing). Research articles describing the development and application of this method are referenced (*11–14*).

6. AIRBORNE ULTRASOUND

The airborne ultrasound test checks seal quality by passing an ultrasound signal through the sealed area of a package or item. The signal strength transmitted through the package seal under test is compared to that previously established for so-called good package seals. Poorly sealed areas will not transmit as much ultrasonic energy as properly sealed areas.

The types of package seals that can be tested using airborne ultrasound include flexible pouch seals and rigid tray seals made of metallic or plastic laminates. This technology can be used to check for the presence and location of package seal defects such as an incomplete seal, a channel, a wrinkle, or extraneous material inclusion, as well as for package seal bond weakness. The method may be used off-line to perform an *x–y* coordinate scan of a seal area, or it may be used to linearly scan seals during on-line manufacturing processes. Airborne ultrasound is a noncontact testing technology that requires no package preparation and no coupling media, such as a liquid or gel, to propagate sound; thus, it is nondestructive to the package under test.

The airborne ultrasound method is described in ASTM F3004 (*15*). The ASTM method test system is composed of a transducer that provides an ultrasonic signal, a means of holding/transporting the item under test within an air gap between the two transducers, and the detection transducer, which captures the intensity of the signal that passed through the air gap and the item under test. The capability of airborne ultrasound to detect specific package seal quality problems will vary on the basis of package material of construction, seal structure (e.g., smooth versus textured surface), scanning speed, and scanning signal strength.

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⟨1208⟩ STERILITY TESTING—VALIDATION OF ISOLATOR SYSTEMS

This chapter provides guidelines for the validation of isolator systems for use in sterility testing of compendial articles. [NOTE—In the context of this chapter, "decontaminated" refers to an item or surface that has been subjected to a process that eliminates viable bioburden.]

Isolators—devices that create controlled environments in which to conduct Pharmacopeial sterility tests—have been used since the mid-1980s. An isolator is supplied with air through a HEPA or better air filter and is able to be reproducibly decontaminated. Closed isolators, which are systems with no direct opening to the external environment, are normally used for sterility testing, although open isolators which allow the egress of materials through a defined opening that precludes the entry of contamination by means of air overpressure may be used. Closed isolators use only decontaminated interfaces or a rapid-transfer port for the transfer of materials. Isolators are constructed of flexible plastics (such as polyvinyl chloride), rigid plastics, glass, or stainless steel.

Isolator systems protect the test article and supplies from contamination during handling by essentially eliminating direct contact between the analyst and the test articles. All transfers of material into and out of the isolator are accomplished in an aseptic fashion while maintaining complete environmental separation. Aseptic manipulations within the isolator are made with half-suits, which are flexible components of the isolator wall that allow the operator a full range of motion within the isolator, or by gloves and sleeves. Operators are not required to wear special clean-room clothing for conducting sterility tests within isolators; standard laboratory clothing is adequate, although a pair of sterile gloves is frequently worn under the isolator gloves as an added precaution against contamination entering the isolator enclosure and for hygiene purposes. The interior of the isolator is treated with sporicidal chemicals that result in the elimination of all viable bioburden on exposed surfaces.

ISOLATOR DESIGN AND CONSTRUCTION

Air Handling Systems

An isolator used for sterility testing is equipped with microbial retentive filters (HEPA filters or better are required). At rest, the isolator meets the particulate air-quality requirements for an ISO Class 5 area as defined in ISO 14644-1 through -3* (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩). However, the isolator need not meet Class 5

* International Organization for Standardization (ISO) International Standards 14644-1, -2, -3, and -7

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conditions during an operation that may generate particulates, and no requirements for air velocity or air exchange rate exist. The isolator should be sealed well enough during decontamination that the dissemination of sporicidal vapors or gases into the surrounding environment is kept to appropriately low levels. When direct openings to the outside environment exist, constant air overpressure conditions maintain sterile conditions within the isolator. In general, both open and closed isolators are maintained at positive pressure relative to the surrounding environment, and overpressures of 20 Pa or more are typical. The user should never exceed the maximum pressure recommended by the isolator manufacturer. Airflow within isolators used for sterility testing is either unidirectional or turbulent.

Transfer Ports and Doors

Isolators may be attached to a "pass-through" decontaminator or transfer isolator to enable the direct transfer of sterile media, sterile dilution fluids, and sterile supplies from the decontaminator into the isolator system. Rapid transfer ports (RTPs) enable two isolators, i.e., the work station and transfer isolator, to be connected to one another, so that supplies can be moved aseptically from one isolator to another. Aseptic connections between two isolators or an isolator and an RTP-equipped container can be made in unclassified environments using RTPs. The nonsterile surfaces of the RTP are connected using locking rings or flanges. A compressed gasket assembly provides an airtight seal, thereby preventing the ingress of microorganisms.

When the two RTP flanges are linked to form an airtight passage, a narrow band of gasket remains that could harbor microbial contamination. This exposed gasket should be routinely disinfected immediately after the connection is made, and before materials are transferred through the RTP. Good aseptic technique is used when transferring materials and care is taken not to touch the gasket with the materials being transferred or with the gloved hands.

Preventive maintenance and lubrication of the gasket assemblies on the flanges is performed according to the RTP manufacturer's recommendations. The RTP gaskets are changed at the recommended frequency and periodically checked for damage, because cut or torn gaskets cannot make a truly airtight seal.

Selection of a Location for the Isolator

Isolators for sterility testing need not be installed in a classified clean room, but it is important to place the isolator in an area that provides limited access to nonessential staff. The appropriate location provides adequate space around the isolator for moving transfer isolators, staging of materials, and general maintenance. No environmental monitoring of the surrounding room is required.

Temperature and humidity control in the room is important to operator safety and comfort and is critical for the effective utilization of certain decontamination technologies. Uniform temperature conditions in the room are desirable when temperature-sensitive decontamination methods are employed. Care should be taken in locating the isolator so that cold spots are avoided that might result in excessive condensation when condensing vapors are used for decontamination.

VALIDATION OF THE ISOLATOR SYSTEM

The isolator system must be validated before its use in sterility testing as part of a batch release procedure. To verify that the isolator system and all associated equipment are suitable for sterility tests, validation studies are performed in three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). The following sections contain points to consider in the validation of isolator systems for sterility testing. The assignment of test functions to a particular phase of the validation program (i.e., IQ, OQ, and PQ) is not critical, as long as proper function of the isolator is demonstrated and documented before its use in compendial *Assays*.

Installation Qualification (IQ)

The IQ phase includes a detailed description of the physical aspects of the system, such as the dimensions, internal configuration, and materials of construction. The unit layout is diagrammed with interfaces and transfer systems clearly and dimensionally indicated. Compliance with design specifications for utility services, such as air supply, vacuum, external exhaust, and temperature and humidity control, is verified. Other equipment used with the isolator system is also described in detail; if any revisions to design specifications are made, these are included. Equipment manuals and copies are catalogued and stored where they can be retrieved and reviewed. Compliance of drawings to design specifications is verified. All drawings and process and instrumentation diagrams are catalogued, stored, and are retrievable.

All documentation is reviewed to verify that it precisely reflects the key attributes of the installed system. This establishes a general benchmark for the isolator system's compliance with design specifications and installation requirements.

Potential process-control or equipment problems that could cause system failure during operation are identified and documented during failure-mode analysis and hazard analysis. The system is modified, if necessary, to minimize the risk of failure, and critical control point methods are established.

The results of the IQ are summarized in an Installation Qualification Report. The following documentation is suggested.

EQUIPMENT

The equipment is listed with its relevant design specifications. The IQ verifies that equipment meeting the appropriate design specifications was received and that it was installed according to the manufacturer's requirements.

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CONSTRUCTION MATERIALS

The construction materials of critical system components are checked for compliance with design specifications. The compatibility of the intended decontamination method with the construction materials is verified.

INSTRUMENTS

System instruments are listed with their calibration status.

UTILITY SPECIFICATIONS

All utilities required for operation—as defined in the operating manuals and process and instrumentation diagrams—are checked for availability and compliance with design specifications. Any connection between utility systems and the isolator system is inspected and conformance of these connections to specifications is verified.

FILTER CERTIFICATION

HEPA filters and other microbial retentive filters are tested and certified; copies of test results and certificates are included in the IQ summary. Purchase orders are reviewed and conformance of the air filtration system to specifications is verified.

COMPUTER SOFTWARE

All computer software associated with the isolator system is listed with its name, size, and file revision number. The master computer disks are checked for proper labeling and stored securely.

Operational Qualification (OQ)

The OQ phase verifies that the isolator system operates in conformance to functional specifications.

OPERATIONAL PERFORMANCE CHECK

This test verifies that all alert and alarm functions comply with their functional specifications. The system's ability to comply with all set points and adjustable parameters is verified.

ISOLATOR INTEGRITY CHECK

The integrity of the isolator is maintained during all normal operating conditions. A leak test is performed to verify the compliance with the manufacturer's functional specifications and to ensure safety prior to charging the isolator with a decontaminating sporicidal chemical. To safeguard against adventitious contamination, isolators are operated at a suitable positive pressure during normal operation. Validation studies must show that the air pressure set point can be maintained and controlled during operation.

DECONTAMINATION CYCLE VERIFICATION

A decontamination cycle that is the function of the decontamination equipment in concert with the isolator(s) is verified.

Different decontamination methods can be used to eliminate bioburden from isolator systems and supplies. Among the chemicals that have been used to treat isolators are peracetic acid, chlorine dioxide, ozone, and hydrogen peroxide; each has different requirements for exposure conditions and process control. It is critical to comply with the manufacturer's operational requirements for the selected decontamination method and to describe them in the functional specifications. The temperature inside the isolator is also important, particularly for hydrogen peroxide vapor decontamination, where it is critical to maintain the concentration relative to the condensation point. Some sterilization chemicals, such as chlorine dioxide and ozone, require the addition of moisture to the isolator prior to decontamination. When elevated relative humidity is required, the ability to control it must be verified during OQ.

It is also important to verify the concentration and distribution of the decontaminating chemical. When applied in gaseous or vapor form, the distribution may be evaluated using chemical indicators, spectroscopic methods, or electronic sensors.

Gas and vapor decontamination methods may require fans in the isolator to distribute the chemical evenly. The location and orientation of these fans are adjusted to ensure optimum air distribution. If the isolator utilizes a recirculating unidirectional airflow system, distribution fans may not be required, but this should be evaluated on a case-by-case basis. Because shelving units, equipment, glove-and-sleeve assemblies, and half-suits have an impact on distribution patterns, distribution checks are done with the isolator fully loaded with equipment and supplies, and the setup of these units is defined and documented.

Many installations use smaller transfer isolators as portable surface decontamination units. In these transfer isolators, test articles and supplies are treated chemically to eliminate bioburden before transfer through an RTP into the testing isolator. Its loading configuration is defined, and configuration drawings are reviewed and verified during the OQ. [NOTE—The decontaminating chemicals used in isolators work on the surfaces of materials; therefore, any surface that is occluded will not be treated and could contain viable bioburden. Special precautions should be in place for treating surfaces known to be occluded with a sporicide if such surfaces may be revealed during the conduct of sterility tests.]

Decontamination agents need to be removed from the isolator after the exposure period, which is accomplished by a current of fresh air provided either by the decontamination equipment or by utilizing the isolator air handling system. Aeration is accomplished either in an open loop, in which the gas is exhausted through a vent to the atmosphere, or in a closed loop, in

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which the chemical is removed and destroyed by the decontamination equipment. The aeration system is checked; if an open-loop configuration is used, the external exhaust system's flow and safety are checked.

DECONTAMINATION CYCLE DEVELOPMENT

When the OQ is completed, decontamination cycle development is performed to establish the parameters necessary for process control during routine decontamination cycles. Any of the methods generally used in the industry for the validation of decontamination processes—including bioburden-based, fractional cycle, and overkill methods—are adequate. The decontamination process is challenged with biological indicators (BIs). The spore population and resistance of the BIs to the decontamination conditions being applied are known. Wherever possible, a D value estimate is done for each B1 system or, alternatively, a survivor curve for the BI system is obtained (see *Biological Indicators—Resistance Performance Tests* ⟨55⟩); it is acceptable to obtain the D value from the BI vendor.

Performance Qualifications (PQ)

The PQ phase verifies that the system is functioning in compliance with its operator requirement specifications. At the completion of the PQ phase, the efficacy of the decontamination cycle and, if appropriate, the adequacy of decontaminating chemical venting are verified. All PQ data are adequately summarized, reviewed, and archived.

CLEANING VERIFICATION

In general, cleaning is not critical for sterility testing applications. However, residual products are a concern in multiproduct testing, particularly for aggressive antimicrobial agents, because these materials could interfere with the ability of subsequent tests to detect low levels of contamination in the product. Concerns about contamination with the product are heightened when it is an inherently antimicrobial powder, because powders are more readily disseminated. Cleaning to a level at which no visible contamination is present is adequate for sterility test isolator systems and is a suitable operator requirement specification. The cleaning method, frequency, equipment, and materials used to clean the isolator are documented.

DECONTAMINATION VALIDATION

The interior surfaces of the isolator, the equipment within the isolator, and the materials brought into the isolator are treated to eliminate all bioburden. The decontamination methods used to treat isolators, test articles, and sterility testing supplies are capable of reproducibly yielding greater than a three-log reduction against highly resistant biological indicators (see *Biological Indicators for Sterilization* ⟨1229.5⟩), as verified by the fraction negative or total kill analysis methods. Total kill analysis studies are suitable for BIs with a population of 10^3 spores per unit, while fraction negative studies are suitable for BIs with a population of 10^5 or greater. A sufficient number of BIs are used to prove statistical reproducibility and adequate distribution of the decontaminating agent. Particular attention is given to areas that pose problems relative to the concentration of the agent. A larger number of BIs may be required in isolators that are heavily loaded with equipment and materials. The ability of the process to reproducibly deliver a greater than three-log kill is confirmed in three consecutive validation studies.

The operator establishes a frequency for re-decontamination of the isolator. The frequency may be as short as a few days or as long as several weeks, depending on the sterility maintenance effort (see *Maintenance of Asepsis within the Isolator Environment*).

PACKAGE INTEGRITY VERIFICATION

Some materials are adversely affected by decontaminating agents, which can result in inhibition of microbial growth. Of concern are the penetration of decontaminating agents into product containers; accessory supplies such as filter sets and tubing; or any material that could come in contact with product, media, or dilution fluids used in the sterility test. It is the responsibility of the operator to verify that containers, media, and supplies are unaffected by the decontamination process. Screw-capped tubes, bottles, or vials sealed with rubber stoppers and crimp overseals have proven very resistant to the penetration of commonly used decontaminating agents. Wrapping materials in metal foil or placing them in a sealed container will prevent contact with the decontaminating agent; however, these procedures may also result in some surfaces not being decontaminated. In some cases, the use of shorter duration decontamination cycles and reduced concentrations may be necessary to minimize penetration of decontaminating agents into the package or container. Cycles that provide a less than three-log kill of resistant BIs may be acceptable provided microbiological analysis of the environment proves that the isolator(s) are free of recoverable bioburden.

In many cases, the operator will choose to treat the surfaces of product containers under test with the decontaminating agent in order to minimize the likelihood of bioburden entering the isolator. It is the responsibility of the operator to demonstrate, via validation studies, that exposure of product containers to the decontaminating agent does not adversely affect the ability of the sterility test to detect low levels of contamination within these test articles. It is suggested that the ability of the package to resist contamination be examined using both chemical and microbiological test procedures. Bacteriostasis and fungistasis validation tests must be performed using actual test articles that have been exposed to all phases of the decontamination process (see *Sterility Tests* ⟨71⟩). This applies to medicinal device packages as well as pharmaceutical container and closure systems.

Validation studies determine whether both sterility test media and environmental control media meet the requirements for *Growth Promotion Test of Aerobes, Anaerobes, and Fungi* under *Sterility Tests* ⟨71⟩.

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MAINTENANCE OF ASEPSIS WITHIN THE ISOLATOR ENVIRONMENT

The ability of the isolator system to maintain an aseptic environment throughout the defined operational period must be validated. In addition, a microbiological monitoring program must be implemented to detect malfunctions of the isolator system or the presence of adventitious contamination within the isolator. Microbiological monitoring usually involves a routine sampling program, which may include, for instance, sampling following decontamination on the first day of operation and sampling on the last day of the projected maintenance of asepsis period. Periodic sampling throughout the use period can be performed to demonstrate maintenance of asepsis within the isolator.

The surfaces within the isolator can be monitored using either contact plates for flat surfaces or swabs for irregular surfaces. However, because media residues could impose a risk on isolator asepsis, these tests are generally best done at the end of the test period. If performed concurrently with testing, care is used to ensure that any residual medium is removed from isolator surfaces, and that those surfaces are carefully cleaned and disinfected. Active air samples and settling plates may be used, but they may not be sufficiently sensitive to detect the very low levels of contamination present within the isolator enclosure.

A potential route for contamination to enter the isolator is during the introduction of supplies and samples into the enclosure. Validating that all materials taken into the isolator enclosure are free of microbial contamination is critical, as is periodic inspection of gaskets to detect imperfections that could allow ingress of microorganisms. Gloves and half-suit assemblies are another potential source of microbial contamination. Gloves are of particular concern because they are used to handle both sterility testing materials and test articles. Resistance to puncture and abrasion should be considered in the selection of gloves and sleeves. Hypalon materials are resistant to both chemical sporicides used in the decontamination of isolators and to punctures and are available in several thicknesses to provide adequate tactile feel through the gloves while maintaining their integrity.

Very small leaks in gloves are difficult to detect until the glove is stretched during use. There are several commercially available glove leak detectors; the operator ensures that the detectors test the glove under conditions as close as possible to actual use conditions. Microbiological tests are used to supplement or substitute physical tests. [NOTE—Standard "finger dab plates" may not be sensitive enough to detect low levels of contamination. Submersion of the gloves in 0.1% peptone water followed by filtration of the diluent and plating on growth media can detect loss of integrity in the gloves that would otherwise go unnoticed.]

Continuous nonviable particulate monitoring within the isolator's enclosure is ideal, because it can quickly detect filter failure. A second choice is periodic monitoring using a portable particle counter. Sampling for particles must be done in a manner that poses no risk to the maintenance of asepsis within the isolator.

INTERPRETATION OF STERILITY TEST RESULTS

A sterility test resulting in a false positive in a properly functioning and validated isolator is very unlikely if bioburden is eliminated from the isolator interior with a high degree of assurance; if gloves, sleeves, and half-suits are free of leaks; and if the RTPs are functioning properly. Nevertheless, isolators are mechanical devices and good aseptic techniques are still required. A decision to invalidate a false positive is made only after fully complying with the requirements of *Observation and Interpretation of Results* under *Sterility Tests* ⟨71⟩.

TRAINING AND SAFETY

As with sterility testing conducted in conventional clean rooms, operators are trained in procedures that are specific to their isolator. Use of proper aseptic techniques is vital to the conduct of sterility tests in isolators, just as it is in clean rooms. Therefore, training in proper aseptic techniques is required for all sterility testing technicians. All training sessions and the evaluation of the operator's performance are documented in the individual's training record. Training of all personnel in the appropriate safety procedures necessary for the operation and maintenance of the isolation system is imperative.

Personnel safety in the use of a decontaminating agent must be assessed. Material Safety Data Sheets, or equivalent documents, are available in the immediate area where the decontaminating agent is being used. All storage and safety precautions are followed. An operational readiness inspection of the safety of the isolator and all associated equipment is performed and documented prior to placing the unit in service.

Add the following:

▲⟨1210⟩ STATISTICAL TOOLS FOR PROCEDURE VALIDATION

1. INTRODUCTION
2. CONSIDERATIONS PRIOR TO VALIDATION
3. ACCURACY AND PRECISION
    3.1 Methods for Estimating Accuracy and Precision
    3.2 Combined Validation of Accuracy and Precision
4. LIMITS OF DETECTION AND QUANTITATION
    4.1 Estimation of LOD
    4.2 Estimation of LOQ
5. CONCLUDING REMARKS

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REFERENCES

1. INTRODUCTION

This chapter describes utilization of statistical approaches in procedure validation as described in *Validation of Compendial Procedures* ⟨1225⟩. For the purposes of this chapter, "procedure validation" refers to the analytical procedure qualification stage of the method life cycle, following design and development and prior to testing.

Chapter ⟨1225⟩ explains that capabilities of an analytical procedure must be validated based on the intended use of the analytical procedure. Chapter ⟨1225⟩ also describes common types of uses and suggests procedure categories (I, II, III, or IV) based on the collection of performance parameters appropriate for these uses. Performance parameters that may need to be established during validation include accuracy, precision, specificity, detection limit [limit of detection, (LOD)], quantitation limit, linearity, and range. In some situations (e.g., biological assay), relative accuracy takes the place of accuracy. This chapter focuses on how to establish analytical performance characteristics of accuracy, precision, and LOD. For quantitative analytical procedures, accuracy can only be assessed if a true or accepted reference value is available. In some cases, it will be necessary to assess relative accuracy. In many analytical procedures, precision can be assessed even if accuracy cannot be assessed. The section addressing LOD can be applied to limit tests in Category II.

The other analytical performance characteristics noted in ⟨1225⟩, which include specificity, robustness, and linearity, are out of scope for this chapter.

Because validation must provide evidence of a procedure's fitness for use, the statistical hypothesis testing paradigm is commonly used to conduct validation consistent with ⟨1225⟩. Although some statistical interval examples are provided in *3. Accuracy and Precision*, these methods are not intended to represent the only approach for data analysis, nor to imply that alternative methods are inadequate.

Table 1 provides terminology used to describe an analytical procedure in this chapter. The definitions for individual determination and reportable value are in alignment with *General Notices, 7.10 Interpretation of Requirements*.

Table 1. Analytical Procedure Validation Terminology

Terminology	Description
Laboratory sample	The material received by the laboratory
Analytical sample	Material created by any physical manipulation of the laboratory sample, such as crushing or grinding
Test portion	The quantity (aliquot) of material taken from the analytical sample for testing
Test solution	The solution resulting from chemical manipulation of the test portion such as chemical derivatization of the analyte in the test portion or dissolution of the test portion
Individual determination (ID)	The measured numerical value from a single unit of test solution
Reportable value	Average value of readings from one or more units of a test solution

Not all analytical procedures have all stages shown in *Table 1*. For example, liquid laboratory samples that require no further manipulations immediately progress to the test solution stage. Demonstration that a reportable value is fit for a particular use is the focus of analytical validation.

Table 2 provides an example of the *Table 1* terminology for a solid oral dosage form.

Table 2. Example for Coated Tablets

Terminology	Description			
Laboratory sample	100 coated tablets			
Analytical sample	20 tablets are removed from the laboratory sample and are crushed in a mortar and pestle			
Test portion	Replicate 1: 1 g of crushed powder aliquot from the analytical sample		Replicate 2: 1 g of crushed powder aliquot from the analytical sample	
Test solution	Replicate 1: Test portion is dissolved in 1 L of solvent		Replicate 2: Test portion is dissolved in 1 L of solvent	
ID	ID 1 of replicate 1: test solution	ID 2 of replicate 1: test solution	ID 1 of replicate 2: test solution	ID 2 of replicate 2: test solution
Reportable value	Average value of four readings			

2. CONSIDERATIONS PRIOR TO VALIDATION

Procedure validation is a cornerstone in the process of establishing an analytical procedure. The aim of procedure validation is to demonstrate that the procedure, when run under standard conditions, will satisfy the requirement of being fit for use. To maximize the likelihood of a successful validation, it is imperative that all aspects of the procedure be well understood prior to the validation. Surprising discoveries (whether "good" or "bad") during validation should be carefully evaluated to determine whether the procedure was adequately developed. Moreover, pre-validation work can reveal suitable approaches to reduce the total size of the validation experiment without increasing the risk of drawing the wrong conclusion. General principles and plans

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for sample preparation, general principles, experimental design, data collection, statistical evaluation, and choice of acceptance criteria should be documented in a validation experimental protocol signed before initiation of the formal validation.

Questions considered prior to validation may include the following:

- What are the allowable ranges for operational parameters, such as temperature and time, that impact the performance of the analytical procedure?
  - Robustness of these ranges can be determined using a statistical design of experiments (DOE).
- What are the ruggedness factors that impact precision?
  - Factors such as analyst, day, reagent lot, reagent supplier, and instrument that impact the precision of a test procedure are called ruggedness factors. When ruggedness factors impact precision, reportable values within the same ruggedness grouping (e.g., analyst) are correlated. Depending on the strength of the correlation, a statistical analysis that appropriately accounts for this dependence may be necessary. Ruggedness factors can be identified empirically during pre-validation or based on a risk assessment.
- Are statistical assumptions regarding data analysis reasonably satisfied?
  - These assumptions may include such factors as normality, homogeneity of variance, and independence. It is useful during pre-validation to employ statistical tests or visual representations to help answer these questions. *Analytical Data—Interpretation and Treatment* ⟨1010⟩ provides information on this topic.
- What is the required range for the procedure?
  - The range of an analytical procedure is the interval between the upper and lower levels of an analyte that has been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written.
- Do accepted reference values or results from an established procedure exist for validation of accuracy?
  - If not, as stated in International Council for Harmonisation (ICH) Q2, accuracy may be inferred once precision, linearity, and specificity have been established.
- How many individual determinations will compose the reportable value, and how will they be aggregated?
  - To answer this question, it is necessary to understand the contributors to the procedure variance and the ultimate purpose of the procedure. Estimation of variance components during pre-validation provides useful information for making this decision.
- What are appropriate validation acceptance criteria?
  - The validation succeeds when there is statistical evidence that the assay is no worse than certain pre-specified levels for each relevant validation parameter.
  - What defines the assay as fit for use, and how does this relate to acceptance criteria?
- How large a validation experiment is necessary?
  - Validation experiments should be properly powered to ensure that there are sufficient data to conclude that the accuracy and precision can meet pre-specified acceptance criteria. Computer simulation is a useful tool for performing power calculations.
  - Efficiencies (both cost and statistical) can be gained if assessment of linearity, accuracy, and precision can be combined.

On the basis of the answers to these and similar questions, one can design a suitable validation experimental protocol.

3. ACCURACY AND PRECISION

A useful model for representing a reportable value is:

$$Y = \tau + \beta + E \quad (1)$$

Y = a reportable value
τ = true or accepted reference value
β = systematic bias of the procedure
E = random measurement error

Both τ (tau) and β (beta) are fixed statistical parameters, and E is a normal random variable with a mean of zero and standard deviation σ (sigma). The magnitude of σ depends on the number of individual readings averaged to obtain the reportable value.

Accuracy of an analytical procedure expresses the closeness of agreement between τ and Y. Closeness is expressed as the long-run average of $(Y - \tau)$. This long-run average is called the systematic bias and is represented with β. To estimate β, it is necessary to know the true value, τ. Chapter ⟨1225⟩ notes that a reference standard or a well-characterized orthogonal procedure can be used to assign the value of τ. Accuracy should be established across the required range of the procedure.

Precision of an analytical procedure is the degree of agreement among reportable values when the procedure is applied repeatedly (possibly under different conditions) to multiple test portions of a given analytical sample. The most common precision metric is the standard deviation σ. This is denoted in *Equation 4* with the variable S. The term σ^2 is called the variance. Precision improves as σ decreases. Many commonly used statistical procedures rely on the assumption of the normal distribution, for which σ is a natural descriptor of variability.

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Change to read:

3.1 Methods for Estimating Accuracy and Precision

An example is provided to demonstrate the test procedure for lot release using statistical analysis. This example uses high-performance liquid chromatography (HPLC). The measured drug substance (DS) is a *USP* compendial substance, so information concerning τ is available (*1*). Three different quantities of reference standard were weighted to correspond to three different percentages of the test concentrations: 50%, 100%, and 150%. The unit of measurement on each reportable value is the mass fraction of DS expressed in units of mg/g and does not change as the level of concentration varies. The value of τ is 1000 mg/g for all three concentrations. The computed statistics from the validation data set include the sample mean ($\overline{Y}$), the sample standard deviation (S), and the number of reportable values (n). *Table 3* presents the $n = 9$ reportable values and the computed statistics.

Table 3. Reportable Values for Experiment

Test Concentration (%)	Test Solution	Reportable Value (mg/g)
50	1	996.07
50	2	988.43
50	3	995.90
100	4	987.22
100	5	990.53
100	6	999.39
150	7	996.33
150	8	993.67
150	9	987.76
Sample mean ($\overline{Y}$)		992.81
Sample standard deviation (S)		4.44

Several assumptions are made for purposes of this example, which allows analysis of the combined data set in *Table 3*:

1. All $n = 9$ reportable values are independent.
2. The standard deviation of the reportable value is constant across all three concentration levels. If this condition is not met, data transformations may still allow combination of all the data in *Table 3* (pooling). If transformations are not successful, each concentration level must be validated for precision separately.
3. The average reportable value is equal across concentration levels. If this condition does not hold, it is necessary to employ an analysis of variance model and validate accuracy for each concentration level separately.

The point estimator for unknown bias β is:

$$\beta = \overline{Y} - \tau \quad (2)$$

β = systematic bias
$\overline{Y}$ = sample mean
τ = true or accepted reference value

where

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} \quad (3)$$

$\overline{Y}$ = sample mean
Y_i = individual values
n = number of reportable values

The point estimator for the unknown value of σ is

$$S = \sqrt{\frac{\sum_{i=1}^{n} \left(Y_i - \overline{Y}\right)^2}{n-1}} \quad (4)$$

S = point estimator for the unknown value of σ

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Y_i = individual values
$\bar{Y}$ = sample mean
n = number of reportable values

Because point estimators have uncertainty associated with them, best practice requires calculation of a statistical confidence interval to quantify the uncertainty. Statistical confidence intervals provide a range of plausible values for β and σ for a given level of confidence. A 100(1 – 2α)% two-sided confidence interval for the bias β is

$$(\bar{Y} - \tau) \pm t_{1-\alpha:n-1} \times \frac{S}{\sqrt{n}} \qquad (5)$$

β = 100(1 – 2α)% two-sided confidence interval of bias
$t_{1-\alpha:n-1}$ = percentile of central t-distribution with area 1 – α to the left and (n – 1) degrees of freedom
S = result found from *Equation 4*
n = number of reportable values

For example, with α = 0.05 and n = 9, $t_{0.95:8}$ = 1.860 provides a 100(1 – 2 × ▲0.05▲ (ERR 1-Mar-2018))% = 90% two-sided confidence interval for β. Using the example data in *Table 3* with τ = 1000 mg/g, the 90% confidence interval on β is

$$(\bar{Y} - \tau) \pm t_{1-\alpha:n-1} \times \frac{S}{\sqrt{n}}$$

$$(992.81 - 1000) \pm 1.86 \times \frac{4.44}{\sqrt{9}} \qquad (6)$$

[–9.94 to – 4.44] mg/g

β = 100(1 – 2α)% two-sided confidence interval of bias
$t_{1-\alpha:n-1}$ = percentile of central t-distribution with area 1 – α to the left and (n – 1) degrees of freedom
S = result found from *Equation 4*

For the standard deviation, one is concerned with only the 100(1 – α)% upper confidence bound since typically, it needs to be shown that the standard deviation is not too large. An upper 100(1 – α)% confidence bound for σ is

$$U = S\sqrt{\frac{n-1}{\chi^2_{\alpha:n-1}}} \qquad (7)$$

U = an upper 100(1 – α)% confidence bound for σ
S = result found from *Equation 4*
n = number of reportable values
$\chi^2_{\alpha:n-1}$ = a percentile of a central chi-squared distribution with area α to the left and (n – 1) degrees of freedom

For example, if α = 0.05 and n = 9, then $\chi^2_{0.05:8}$ = 2.73. Using the data in *Table 3*,

$$U = S\sqrt{\frac{n-1}{\chi^2_{\alpha:n-1}}}$$

$$U = 4.44\sqrt{\frac{9-1}{2.73}} = 7.60 \text{ mg/g} \qquad (8)$$

The confidence intervals in *Equations 5* and *7* can be used to perform statistical tests against criteria included in the validation protocol. Use of point estimates only does not provide the required scientific rigor. In particular, the two-sided confidence interval in *Equation 5* can be used to perform a two one-sided test (TOST) of statistical equivalence (*2*). Assume in the present example that the accuracy requirement is validated if evidence demonstrates that the absolute value of β is NMT 15 mg/g. Since the computed confidence interval from –9.94 to –4.44 mg/g falls entirely within the range from –15 to +15 mg/g, the bias criterion is satisfied. Most typically, the TOST employs a type I error rate of α = 0.05. This error rate represents the maximum risk of declaring that the acceptance criterion is satisfied, when in truth it is not satisfied. Thus, with α = 0.05, the two-sided confidence interval in *Equation 5* is 100(1 – 2α)% = 90%.

The upper bound in *Equation 7* is used to validate precision. Suppose the pre-defined acceptance criterion for precision requires σ to be <20 mg/g. The computed upper bound of 7.60 mg/g in *Equation 8* represents the largest value we expect for σ with 95% confidence. Since 7.60 mg/g is <20 mg/g, precision has been successfully validated with a confidence of 95%.

Change to read:

3.2 Combined Validation of Accuracy and Precision

When assessing whether an analytical procedure is fit for its intended purpose, it is often useful to consider the combined impact of bias and precision. The degree to which β impacts the usefulness of an analytical procedure depends in part on σ.

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That is, a procedure with a relatively small value of σ can accommodate a relatively greater value of β than a procedure with a greater value of σ. For this reason, it is useful to establish a single criterion that can be used to simultaneously validate both accuracy and precision. One such criterion is proposed in a series of articles by Hubert et al. (*3–5*) and seeks to ensure that

$$Pr(-\lambda < Y - \tau < \lambda) \geq P, \text{ or}$$

$$Pr(-\lambda + \tau < Y < \lambda + \tau) \geq P \quad (9)$$

Pr = reference probability
λ = acceptable limit
Y = a reportable value
τ = true or accepted reference value
P = desired probability value

Equation 9 has a dual interpretation. It can be interpreted as either (i) the probability that the next reportable value falls in the range from $(-\lambda + \tau)$ to $(\lambda + \tau)$ is $\geq P$, or (ii) the proportion of all future reportable values falling between $(-\lambda + \tau)$ and $(\lambda + \tau)$ is $\geq P$. Accordingly, two statistical intervals have been proposed to demonstrate that *Equation 9* is true:

1. A prediction interval (also referred to as an expectation tolerance interval) is used to demonstrate (i).
2. A tolerance interval (also referred to as a content tolerance interval) is used to demonstrate (ii).

Hahn and Meeker (*6*) note that the prediction interval is also referred to as an expectation tolerance interval and that the tolerance interval is also referred to as a content tolerance interval. Because the inference associated with the tolerance interval concerns a larger set of values, the tolerance interval is always wider than the prediction interval. Selection of an interval will depend on the desire to validate ▲▲ (ERR 1-May-2018) either (i) or (ii) and a company's risk profile.

Either interval can be used in the following manner to evaluate accuracy and precision simultaneously through *Equation 9*.

1. Compute the appropriate statistical interval using *Equation 10* for the prediction interval and *Equation 11* for the tolerance interval.
2. If the computed interval falls completely in the range from $(-\lambda + \tau)$ to $(\lambda + \tau)$, the criterion in *Equation 9* is satisfied, and the procedure is validated for both accuracy and precision.

The prediction interval used to validate *Equation 9* is

$$\overline{Y} \pm t_{(1+P)/2;n-1} \times S\sqrt{1+\frac{1}{n}} \quad (10)$$

$\overline{Y}$ = sample mean
$t_{(1+P)/2;n-1}$ = percentile of a central *t*-distribution with area $(1 + P)/2$ to the left and $(n - 1)$ degrees of freedom
S = result found from *Equation 4*

The 100(1 − α)% tolerance interval used to validate *Equation 9* is

$$\overline{Y} \pm K \times S$$

$$K = \sqrt{\frac{Z^2_{(1+P)/2} \times (n-1)}{\chi^2_{\alpha;n-1}} \times \left(1+\frac{1}{n}\right)} \quad (11)$$

$\overline{Y}$ = sample mean
K = result found from *Equation 11*
S = result found from *Equation 4*
$Z^2_{(1+P)/2}$ = ▲the square of the standard normal▲ (ERR 1-May-2018) percentile with area $(1 + P)/2$ to the left
n = number of reportable values
$\chi^2_{\alpha;n-1}$ = a chi-squared percentile with area α to the left and (n − 1) degrees of freedom

The formula for K is based on an approximation by Howe (*7*), although exact tabled values can be found in several sources. The approximation works well in practical situations if exact values are not available.

For the data in *Table 3* with $P = 0.90$, the interval for *Equation 10* is computed as

$$\overline{Y} \pm t_{(1+P)/2,n-1} \times S\sqrt{1+\frac{1}{n}}$$

$$992.81 \pm 1.86 \times 4.44\sqrt{1+\frac{1}{9}} \quad (12)$$

$$[984.1 \text{ to } 1001.5] \text{ mg/g}$$

The interval for *Equation 11* with $1 - \alpha = 0.90$ and $P = 0.90$ is

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$$K = \sqrt{\frac{Z^2_{(1+P)/2} \times (n-1)}{\chi^2_{\alpha,n-1}} \times \left(1+\frac{1}{n}\right)}$$

$$K = \sqrt{\frac{(1.64)^2 \times (9-1)}{3.49} \times \left(1+\frac{1}{9}\right)} = 2.63$$

$$\bar{Y} \pm K \times S$$

$$992.81 \pm 2.63 \times 4.44$$

[981.2 to 1004.5] mg/g (13)

The exact value for K is 2.637, and the approximation is seen to work quite well. As predicted earlier, the interval for *Equation 13* is wider than the interval for *Equation 12*.

Suppose the criterion for *Equation 9* is designed to ensure that the difference between Y and τ is <2% of τ with a probability NLT $P = 0.90$. Thus,

$$-\lambda + \tau = \tau(-0.02 + 1) = 1000(0.98) = 980 \text{ mg/g}$$

$$\tau + \lambda = \tau(1 + 0.02) = 1000(1.02) = 1020 \text{ mg/g} \quad (14)$$

Since both *Equations 12* and *13* fall in the range from 980 to 1020 mg/g, the procedure is validated using either interval.

It is also possible to estimate $Pr(-\lambda < Y - \tau < \lambda)$ in *Equation 9* directly using either the confidence interval described by Mee (*8*) or a Bayesian approach. The validation criterion is thus satisfied if this estimated probability exceeds P. A Bayesian tolerance interval is provided in Wolfinger (*9*) and can be computed using the statistical software package WinBUGS (*10,11*). Bayesian analyses can be challenging, and the aid of an experienced statistician is recommended.

4. LIMITS OF DETECTION AND QUANTITATION

The LOD and limit of quantitation (LOQ) are two related quantities determined in the validation of Category II procedures of ⟨1225⟩. These are procedures for the determination of impurities or degradation products in DS and finished pharmaceutical products. Only one is needed for each use: LOQ for quantitative tests and LOD for qualitative limit tests. These limits are also known under other names, including detection limit (DL) for LOD and lower limit of quantitation (LLOQ) for LOQ.

The following definitions are consistent with ⟨1225⟩ and ICH Q2:

- The LOD is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.
- The LOQ is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

Candidate values for LOD or LOQ are examined during pre-validation or based on a risk assessment. The candidate values must then be verified. This is particularly important for LOQ, since the formulas for determining candidate values do not address the acceptable accuracy and precision requirement. Verification of the candidate values is performed as part of the validation protocol.

4.1 Estimation of LOD

The basic approach to estimating LOD is based on an alternative definition adopted by the International Union of Pure and Applied Chemistry (IUPAC) and the International Organization for Standardization (ISO). This definition introduces the notion of false-positive and false-negative decisions, thus recognizing the risk elements in using the LOD for decision making, and the definition makes clear that these values are dependent on laboratory capability.

The IUPAC/ISO definition of LOD is based on the underlying concept of a critical value (R_C), defined as the signal readout exceeded with probability α when no analyte is present. That is,

$$R_C = B + Z_{1-\alpha}\sigma_E \quad (15)$$

R_C = signal readout exceeded with probability α when no analyte is present
B = estimated mean readout for blanks
$Z_{1-\alpha}$ = a standard normal quantile with area $1 - \alpha$ to the left
σ_E = true repeatability deviation

Figure 1 presents this relationship graphically.

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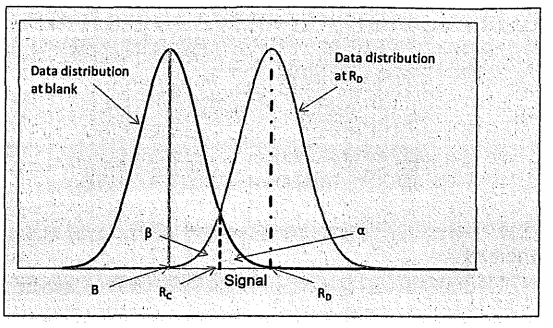

Figure 1. Determination of R_C and R_D.

For example, if $\alpha = 0.05$, $1 - \alpha = 0.95$ and $Z_{0.95} = 1.645$. This determination depends on the distribution of values obtained when analyzing blanks. The LOD in the signal space (R_D) is defined as that value, which if true, is such that R_C is exceeded with probability $1 - \beta$. That is,

$$R_D = R_C + Z_{1-\beta}\sigma_E \quad (16)$$

R_D = LOD in the signal space
R_C = critical value using IUPAC/ISO definition of LOD
$Z_{1-\beta}$ = standard normal quantile with area $1 - \beta$ to the left
σ_E = true repeatability deviation

Solving *Equations 15* and *16* for R_D, we have

$$R_D = B + (Z_{1-\alpha} + Z_{1-\beta})\sigma_E \quad (17)$$

R_D = LOD in the signal space
B = estimated mean readout for blanks
$Z_{1-\alpha}$ = a standard normal quantile with area $1 - \alpha$ to the left
$Z_{1-\beta}$ = a standard normal quantile with area $1 - \beta$ to the left
σ_E = true repeatability deviation

Note that this definition allows for two values to be selected by the laboratory: α and β (which need not be equal). The α represents the type I or false-positive error rate, and β represents the type II or false-negative error rate. In *Figure 1*, R_C and R_D are illustrated with $\alpha = \beta = 0.05$ for normally distributed data so that $Z_{1-\alpha} = Z_{1-\beta} = 1.645$. Although the values of α and β need not be equal, this choice leads to a common rule for R_D, namely $B + 3.3\sigma_E$ ($3.3 \cong 2 \times 1.645$).

The LOD on the concentration scale is then found by converting the value in the signal scale, R_D, to one in the concentration scale, LOD, as shown in *Figure 2*.

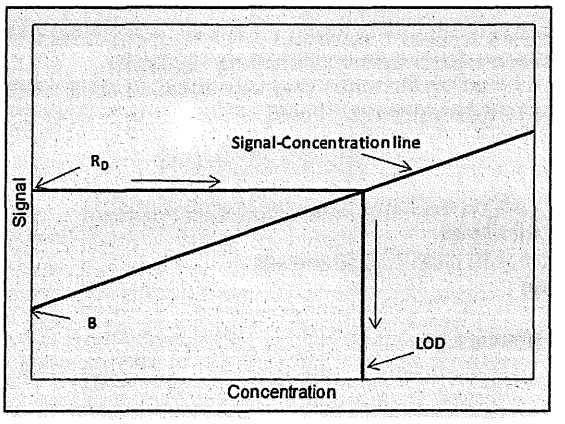

Figure 2. Determination of LOD from R_D.

This step requires that the signal (R) versus concentration (X) line, $R = B + mX$, as well as σ_E, be known exactly. The formulation provided in this section assumes the regression measurements are independent.

The LOD on the concentration scale is then calculated as

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$$LOD = \frac{R_D - B}{m} = \frac{(Z_{1-\alpha} + Z_{1-\beta})\sigma_E}{m} \quad (18)$$

LOD = limit of detection
R_D = LOD in the signal space
B = estimated mean readout for blanks
$Z_{1-\alpha}$ = standard normal quantile with area 1 – α to the left
$Z_{1-\beta}$ = standard normal quantile with area 1 – β to the left
σ_E = true repeatability deviation
m = slope

As a statistical procedure, the LOD definition in *Equation 18* is unsatisfactory for two reasons. First, since σ_E is generally unknown, it must be determined how to best estimate this parameter. This is complicated because σ_E is typically concentration dependent. Two common estimates are (i) the standard deviation of the blank responses and (ii) the standard deviation obtained from deviations about the regression line of signal on concentration. The choice needs to be the value that best represents σ_E in the neighborhood of the LOD. Laboratories will often pick a worst-case value for σ_E. If the LOD is still suitable for its intended use, the laboratories are protected against understating the LOD. Understatement of the LOD results in an inflated type II error rate (β) and a deflated type I error rate (α).

The second statistical concern with *Equation 18* is how to incorporate uncertainty due to the fact that the exact slope of the regression line of signal on concentration is unknown. Because the regression line is estimated, the definition of R_D in *Equation 17* is itself an estimate. This is corrected by using a statistical prediction interval that takes into account the uncertainty in the estimated line as well as the variability associated with a future observation. The expanded formula for the critical value, R_C, originally defined in *Equation 15* that accounts for this uncertainty is

$$R_c = B + t_{1-\alpha:n-2} \times S \sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n}(X_i - \overline{X})^2}} \quad (19)$$

$$S = \sqrt{\frac{\sum_{i=1}^{n}(R_i - B - mX_i)^2}{n-2}}$$

R_C = critical value using the IUPAC/ISO definition of LOD
B = intercept of the fitted calibration line
$t_{1-\alpha:n-2}$ = percentile of a central t-distribution with area 1 – α to the left and (n – 2) degrees of freedom
S = standard error of regression line
$\overline{X}$ = average concentration
n = number of observations used in the regression analysis
X_i = concentration value used in determining the line
R_i = reference interval
m = slope

Equation 19 differs from *Equation 15* because the t-distribution is used instead of the normal distribution for the multiplier, and two additional terms appear in the square root to capture the uncertainty of the regression line.

A second equation for R_C answers the question, "Above which concentration can we be confident that we will obtain signals that are distinguishable from background?" This question is answered by using the lower 100(1 – β)% prediction bound of the calibration curve as shown in *Figure 3*.

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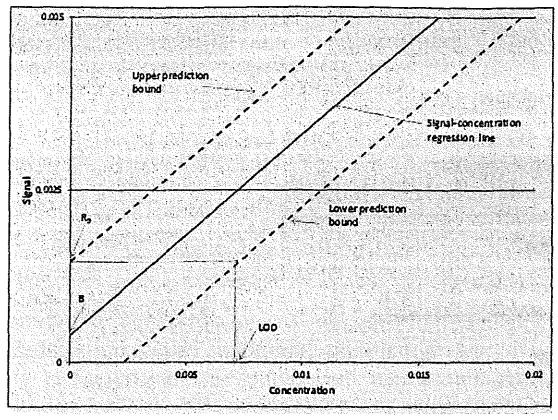

Figure 3. Determination of LOD using prediction bounds.

Figure 3 is similar to *Figure 2*, but uses two dashed curves instead of the solid calibration line. Here

$$R_C = B + LOD \times m - t_{1-\beta:n-2} \times S \sqrt{1 + \frac{1}{n} + \frac{(LOD - \overline{X})^2}{\sum_{i=1}^{n}(X_i - \overline{X})^2}} \qquad (20)$$

R_C = critical value using the IUPAC/ISO definition of LOD
B = estimated intercept of the fitted calibration line
LOD = limit of detection
m = slope
$t_{1-\beta:n-2}$ = percentile of a central t-distribution with area $1 - \beta$ to the left and $(n - 2)$ degrees of freedom
S = standard error of regression line
$\overline{X}$ = average concentration value squared
n = number of observations used in the regression analysis
X_i = concentration value used in determining the line

After equating *Equation 19* and *Equation 20*, and cancelling the B terms,

$$t_{1-\alpha:n-2} \times S \sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n}(X_i - \overline{X})^2}} = LOD \times m - t_{1-\beta:n-2} \times S \sqrt{1 + \frac{1}{n} + \frac{(LOD - \overline{X})^2}{\sum_{i=1}^{n}(X_i - \overline{X})^2}} \qquad (21)$$

$t_{1-\alpha:n-2}$ = percentile of a central t-distribution with area $1 - \alpha$ to the left and $(n - 2)$ degrees of freedom
S = standard error of regression line
$\overline{X}^2$ = average concentration value squared
LOD = limit of detection
m = slope
$t_{1-\beta:n-2}$ = percentile of a central t-distribution with area $1 - \beta$ to the left and $(n - 2)$ degrees of freedom
n = number of observations used in the regression analysis
X_i = concentration value used in determining the line
$\overline{X}$ = average concentration value

Equation 21 is a quadratic equation for LOD that can be solved exactly or by using iterative search tools available in spreadsheets. A slightly conservative (overly large) approximation for LOD that does not require a quadratic solution is obtained by assuming that LOD is negligible compared to $\overline{X}$ [i.e., $(LOD - \overline{X})^2$ is replaced with $\overline{X}^2$]. The resulting equation under this simplification is

$$LOD = (t_{1-\alpha:n-2} + t_{1-\beta:n-2}) \times \frac{S}{m} \sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n}(X_i - \overline{X})^2}} \qquad (22)$$

LOD = limit of detection
$t_{1-\alpha:n-2}$ = percentile of a central t-distribution with area $1 - \alpha$ to the left and $(n - 2)$ degrees of freedom
$t_{1-\beta:n-2}$ = percentile of a central t-distribution with area $1 - \beta$ to the left and $(n - 2)$ degrees of freedom
S = standard error of regression line
$\overline{X}$ = average concentration value

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m = slope
n = number of observations used in the regression analysis
X_i = concentration value used in determining the line

which is similar in form to *Equation 18*. *Equations 18* and *22* both allow the two error probabilities, α and β, to differ. Often they are both taken as equal to 0.05.

The data in *Table 4* are used to demonstrate calculation of the LOD.

Table 4. Data for LOD Example

Concentration X (mg/mL)	Area (signal)
0.01	0.00331
0.02	0.00602
0.05	0.01547
0.10	0.03078
0.15	0.04576
0.25	0.07592

Fitting the linear regression to these data yields the regression line:

$$\text{Area} = 0.000235 + 0.3032 \times \text{Concentration} \quad (23)$$

so that $m = 0.3032$ and $B = 0.000235$. Values needed to compute LOD shown in *Equation 22* with $\alpha = \beta = 0.05$ are provided in *Table 5*.

Table 5. Statistics Needed to Compute LOD in Concentration Units

Statistic	Value
n	6
m (slope)	0.3032
S	0.00019
$t_{1-\alpha:n-2} = t_{0.95:4}$	2.132
$t_{1-\beta:n-2} = t_{0.95:4}$	2.132
$\bar{X}$	0.0967
$\sum_{i=1}^{n}\left(X_i - \bar{X}\right)^2$	0.0419

The value of LOD computed from *Equation 22* is

$$LOD = (t_{1-\alpha:n-2} + t_{1-\beta:n-2}) \times \frac{S}{m}\sqrt{1 + \frac{1}{n} + \frac{\bar{X}^2}{\Sigma_{i=1}^{n}(X_i - \bar{X})^2}}$$

$$LOD = (2.132 + 2.132) \times \frac{0.00019}{0.3032}\sqrt{1 + \frac{1}{6} + \frac{0.0967^2}{0.0419}} \quad (24)$$

$$LOD = 0.0032 \text{ mg/mL}$$

LOD = limit of detection
$t_{1-\alpha:n-2}$ = percentile of a central t-distribution with area $1 - \alpha$ to the left and $(n - 2)$ degrees of freedom
$t_{1-\beta:n-2}$ = percentile of a central t-distribution with area $1 - \beta$ to the left and $(n - 2)$ degrees of freedom
S = residual standard error found from *Equation 4*
$\bar{X}^2$ = average concentration squared
m = slope
n = number of observations used in the regression analysis
X_i = concentration value used in determining the line

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4.2 Estimation of LOQ

As previously discussed, the important consideration in determining the LOQ is the estimation of what LOQ is required based on the intended use. The validation is designed to validate accuracy and precision in the neighborhood of the required LOQ.

In the absence of such knowledge, or where the laboratory wants to determine how low the LOQ might be (e.g., for potential other uses), then the laboratory can start with potential LOQ values greater than but near the LOD. Alternatively, methods for determining the LOD can be adapted to the LOQ as candidate starting values. Essentially, the formula used to compute LOD in *Equation 22* can be used to compute LOQ by replacing $(t_{1-\alpha;n-2} + t_{1-\beta;n-2})$ with 10. Values other than 10 can be used if justified. Once candidate values are obtained (typically during pre-validation), accuracy and precision are validated at these values.

All of the methods presented in this section are based on two assumptions: linearity and homogeneity of variance across the range of concentrations used in determining the calibration curve. Neither is a necessary condition. The calibration curve may be nonlinear, and a weighted least squares approach can be used to account for a lack of homogeneity. If the curve is nonlinear or the concentration variances vary greatly in the range of the LOD and LOQ, it is best to seek expert statistical help in defining LOD and LOQ. If variability about a straight line exists but is not large, an unweighted regression of the calibration curve will provide an average variability that can be used in the LOD and LOQ formulas.

Procedures other than those described above, such as signal-to-noise ratios, can be used to estimate LOD and LOQ. In either case, analysts should consider these values as preliminary and proceed to verify them, particularly if they fall below the concentration values used in determining the calibration curve. Verification involves analyzing samples with concentrations near the preliminary LOD and LOQ. Consideration should be given to how low must an LOD and LOQ be such that the procedure is suitable. For example, if data are already available at a level below the required LOD and a signal was detectable at that lower value, then that lower value may be taken as a verified LOD. There is little value in further verification—given the current requirement. There could still be value in verification of a lower value in case the requirement changes.

5. CONCLUDING REMARKS

This chapter presented some simple statistical methods that can be used in procedure validation as described in ⟨1225⟩. These methods may not be applied in all situations, and other statistical approaches, both more and less sophisticated, may be appropriate for any particular situation.

Re-evaluation of a procedure should be considered whenever use of the procedure changes. For example, if a new product strength is introduced, the procedure is transferred to a new lab, samples are to be tested following a new type of stress test, or specifications change, a re-validation is most likely appropriate. In some situations, a re-assessment of existing data to revised acceptance limits is sufficient.

Finally, although not part of procedure validation, it is recommended that some type of statistical process control be used to monitor the performance of the procedure. Such a process provides early warning of "drift" in the analytical performance characteristics of accuracy and precision. Such changes in performance are not uncommon, and often occur as a result of worn equipment, change of routines, or aging reagents.

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▲ (USP41)

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Change to read:

⟨1211⟩ ▲STERILITY ASSURANCE▲ (USP41)

Change to read:

▲INTRODUCTION

This informational chapter provides general information on the concepts and principles involved in the preparation of materials that must be sterile. Within the strictest definition of sterility, an item is deemed sterile only when it contains no viable microorganisms. However, this textual definition cannot be applied to actual items labeled as sterile because of irresolvable limitations in testing. Sterility cannot be demonstrated without the destructive testing of every sterile unit. In a real sense, microbiological safety is achieved through the implementation of interrelated controls that in combination provide confidence that the items are suitable for use as labeled. It is the controls that provide the desired assurance from microbiological risk rather than the results of any in-process or finished goods testing. The verification of safety of products labeled sterile is generally known as "sterility assurance" and that nomenclature will be used throughout this chapter.

The establishment of an effective sterility assurance program requires information about the material to be sterilized. An initial determination should be made regarding the potential for terminal sterilization of the material in its primary container applying the principles defined in *Sterilization of Compendial Articles* ⟨1229⟩. As described, the appropriate process provides a balance between conditions that are lethal to potential bioburden present in/on the item and those that preserve its essential quality attributes. Depending upon the results of that determination, sterility of the item may be achieved by either aseptic processing or terminal sterilization. The potential for a process that relies on both the protective measures inherent to aseptic processing and the lethal nature of terminal sterilization may offer advantages. Regardless of the process selection decision, the establishment of design, operation, process controls, and monitoring systems is essential to provide the necessary confidence in the outcome.

The production of sterile products is subject to numerous factors that influence the outcome (see *Figure 1*). The identified factors in the image should be considered for their impact on sterility of the final product, although not all of the influencing elements are depicted.

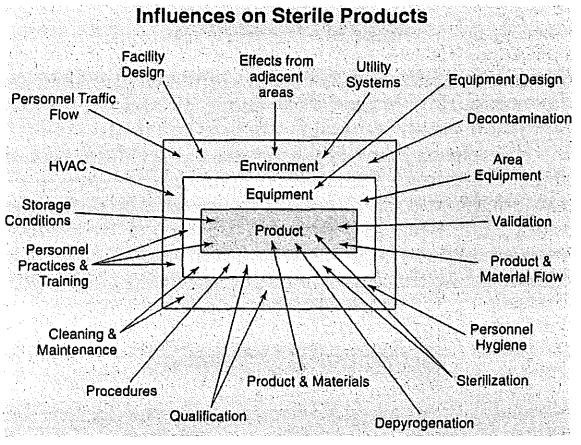

Figure 1. Influences on sterile products.

The decisions made relative to the influences shown in *Figure 1* will determine the success of the sterility assurance program. Poor choices, regardless of any successful process controls associated with them, must be acknowledged as fundamentally unsound. The process design objective is related to contamination controls intended to obviate risk of microbial ingress. This focus is appropriate regardless of whether the process used is aseptic processing or terminal sterilization.

Recognition that operating personnel are the most significant contributors of microbiological risk leads to design preferences and operating principles that should be adhered to with respect to sterile operations. This knowledge underscores the importance of separating personnel from the aseptic environment and limiting their interaction with sterilized components and product(s). The means for accomplishing these goals are embodied in two complementary practices (*1*):

- The use of automation technology—to reduce or eliminate personnel interventions and thus personnel-borne contamination
- The use of separative technologies—to eliminate, to the extent technically possible, human sourced contamination

Thus the implementation of appropriate contamination control procedures is paramount in design and operation of sterile product manufacturing systems.

Consideration of these principles adapts the Quality by Design (QbD) approach widely adopted in regulatory standards (*2–3*). Using QbD concepts in sterile operations is markedly different from the applications in the typical formulation, pharmaceutical, chemical, or biological synthesis process. The establishment of direct linkage between a monitored condition and process outcome with respect to sterile manufacturing is statistically difficult and analytically uncertain. The situation with

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respect to the definition of physical design elements is similar. Given the great variation in sterile product manufacturing with respect to scale, configuration, and complexity, it follows that the design alternatives and operating practices must also be flexible. Thus, the recommendations provided in this chapter are entirely non-numeric, because there are no ready means with which to demonstrate the suitability of specific values. Instead, QbD for sterile processing should be driven toward a singular goal of optimizing contamination control with a particular focus on the microbial risk impact of personnel. The specific means vary but should be of prime consideration in process design. *Figure 2* outlines the elements that contribute to sterility assurance, as described in the remaining sections of this chapter.

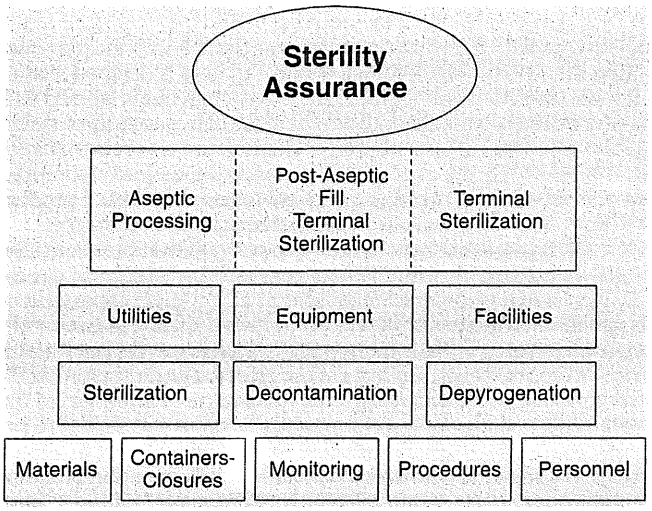

Figure 2. Elements contributing to sterility assurance.

Aseptic Processing

There are a substantial number of sterile products that cannot be terminally sterilized because of adverse impact on the product/package's essential properties and must be prepared by aseptic processing. Aseptic processes are designed to prevent the introduction of viable microorganisms into/onto separately sterilized materials during their assembly into a sealed sterile package. Aseptic processes can vary in complexity from comparatively simple filling/sealing to challenging and lengthy manufacturing sequences required for complex items. Regardless of process scale, all of the individually sterilized materials must be protected from contamination from the point of sterilization through closure of the primary package. This is accomplished through adherence to the principles described below in which an International Organization for Standardization (ISO) 5 condition is maintained when materials are exposed to the environment (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩). Exclusive to aseptic processing is the execution of process simulations that support batch or campaign duration (*4–6*).

Terminal Sterilization

Terminally sterilized products are the lowest risk category of sterile pharmaceutical products. Unlike products aseptically manufactured under conditions designed to prevent microbial ingress, terminally sterilized products are subjected to a sterilization process that imparts a quantifiable safety level. Terminal sterilization processes achieve this by delivering measurable physical conditions that correspond to microbial lethality. For terminally sterilized products, sterility assurance is defined in terms of the probability of nonsterility (PNS), or the probability of the terminal sterilization process generating a nonsterile unit (PNSU). Terminal sterilization processes must achieve a consistent validated performance of a PNSU of $\leq 10^{-6}$ (a probability of NMT 1 nonsterile unit in 1 million units produced) (see ⟨1229⟩). The convention by which terminal sterilization cycles are developed and validated ensures that the actual PNSU is typically much lower (better) than the minimum standard of $<10^{-6}$.

Chapter ⟨1229⟩ summarizes the common requirements for sterilization process design, development, validation, and process control. Terminal sterilization processes share common requirements of well-defined process parameters strictly controlled within defined operating limits. Terminal sterilization must be supported by a system of product disposition, which includes the assessment of critical physical process parameters, presterilization product parameters (e.g., bioburden, container–closure integrity), and environmental parameters. Terminal sterilization can rely on parametric release practices to obviate the need for sterility testing (see Terminally Sterilized Pharmaceutical Products—Parametric Release ⟨1222⟩).

Post-Aseptic Processing Terminal Sterilization

An aseptic process followed by a terminal sterilization process provides superior control over the presterilization bioburden, such that the subsequent sterilization process can be designed with less overall lethality, thereby making it possible to substantially extend the use of terminal sterilization to products with greater sensitivity to the applied energy of the process. From a patient safety perspective, this approach has the following distinct advantages:

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- An adventitious contaminant introduced during aseptic processing is easily killed by the terminal sterilization step, reducing the extent of in-process environmental monitoring performed
- Bioburden controls for the terminal process are simplified because all units have been aseptically filled
- Where a product is made using either process alone, the limitations of each (no terminal lethal component in aseptic, more degradation in terminal sterilization) would persist
- Where bioburden is controlled through aseptic processing, terminal sterilization can be applicable at lower lethality levels

Classical F_0, time-temperature, and radiation dose (kGy) targets for sterilization processes are arbitrarily selected and intended to simplify process validation, but in reality serve to reduce the use of terminal treatments. Physical lethality data based on fixed numerical values are inherently conservative and disregard the degradative impact of the sterilization process on the product. The focus must be on the ability of the process to kill bioburden organisms rather than biological indicators (see ⟨1229⟩ and *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩).

While product quality attributes can be impaired by "standard" sterilizing conditions, the combined process can utilize less aggressive sterilizing conditions to minimize adverse effects upon the product and primary packaging materials. If the terminal treatment follows aseptic processing, then the sterilizing conditions need not be excessive as there is essentially no risk from presterilization bioburden in the filled containers. Sterilization process conditions would be dictated by the specifics of the product in parallel with the establishment of appropriate controls on presterilization bioburden derived from environmental and prefiltration isolates.

Containers and Closures

The container and closure for a sterile formulation are integral parts of the sterile product. The container materials provide essential protection to the product throughout its shelf life and are chosen to minimize interaction with, preserve the quality attributes of, and facilitate dispensing of the sterile product. The container materials should be readily sterilizable, either separately prior to filling and/or together with the formulation in a terminal process. Containers and closures should be selected for:

- Reliability of container–closure integrity over the shelf life
- Absence of interaction with formulation materials
- Ease of handling in the processing environment and during administration
- Tolerance of variation in equipment and other components
- Cleanliness including freedom from particulates and absence of leachable or extractable chemicals
- Compatibility with the product
- Control over endotoxin content (where appropriate)
- Protection of components prior to use (where appropriate)
- Compatibility with preparation, sterilization, and depyrogenation processes (where appropriate)

The essential aspects of container–closure materials for sterile products are subject to numerous requirements elsewhere within *USP–NF*. The reader must consider the content provided in *Injections and Implanted Drug Products* ⟨1⟩, *Bacterial Endotoxins Test* ⟨85⟩, *Visible Particulates in Injections* ⟨790⟩, *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Depyrogenation* ⟨1228⟩, and ⟨1229⟩.

Decontamination

Decontamination is a broadly defined term used to describe a variety of processes that reduce microbial populations without an expectation for total kill.[1] It is not a substitute for sterilization; a sterilization process should be used wherever possible. A variety of chemical agents and methods are used that vary depending upon the application. Decontamination is used for bioburden reduction of materials, equipment, and environments in support of sterile product manufacture:

- For materials and surfaces that cannot be sterilized
- For materials and surfaces that do not require sterilization

Decontamination processes are ordinarily separated into two major categories based upon their effectiveness against spore-forming microorganisms (see *Disinfectants and Antiseptics* ⟨1072⟩). Sporicidal treatments are used in critical applications such as isolator decontamination, air-lock/pass-throughs, etc. Their toxicity to personnel and sometimes corrosive chemistry may preclude their exclusive use for microbial control. Non-sporicidal agents have fewer safety and material impact concerns, and the occasional use of a sporicidal agent is required to control spore populations. Applications for decontamination are diverse; among the more common uses are:

Decontamination of controlled environments and non-product contact surfaces

- In conventional cleanrooms, including restricted access barrier systems (RABS), this is predominantly a manual process performed after cleaning of the room/production line
- Decontamination of items upon transition into an environment of higher classification
- Isolators commonly use an automated process
- Periodic decontamination of operator gloves during processing

Decontamination of product contact surfaces

[1] Sterilization is preferred over decontamination and should be utilized wherever possible, consistent with minimization of handling post-sterilization.

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- Large equipment (e.g., stopper bowls) can be manually sanitized on a frequent basis in addition to sterilization to avoid the extensive manipulation required for their installation post-sterilization[2]
- Re-decontamination of sterilized equipment after aseptic assembly or intervention
- Periodic decontamination of previously sterilized utensils prior to interventions

Depyrogenation

The minimization of pyrogen content is a requirement for injectable products. During the production of sterile products, depyrogenation processes are used in a variety of ways to minimize pyrogenic contamination of surfaces, materials, and products. Details on depyrogenation processes are provided in ⟨1228⟩.

Equipment

Equipment used for sterile product manufacturing varies in its impact on the manufacturing process and on product quality and should have several important characteristics. For example, the equipment should:

- Operate reliably and produce products of consistent quality
- Not adversely impact essential product quality attributes
- Be easily cleanable and sterilizable, as necessary
- Minimize human intervention during set-up and operation through such features as physical separation, automation, and robotics
- Be tolerant of variations in container–closure materials
- Designed to minimize product exposure to the background environment

The extent to which the equipment interacts with process materials and the product affects the level of impact. Process equipment can influence the quality of the finished product in a variety of ways, and this can occur prior to and after sterilization and depyrogenation.

EQUIPMENT IN DIRECT CONTACT WITH COMPONENTS, CONTAINERS, CLOSURES, AND STERILE PRODUCTS

This equipment category includes those items in direct contact with the drug substance, drug product, raw materials, and primary packaging components, including, for example, mixing and storage vessels, piping systems and tubing, filters, filling pumps, lyophilizer shelves, and feed hoppers. Product contact surfaces of this equipment are designed and may require additional treatment to minimize adverse impact (microbial, particulate, and chemical) on the contacted materials. The procedures used for the cleaning and sterilization of direct contact surfaces, including dirty, clean, and sterile hold times, must be validated to ensure they do not adversely impact essential product quality attributes as well as to verify the effectiveness of the cleaning procedure and that no microbial recontamination/proliferation occurs during equipment storage. Direct contact utensils are subject to the same considerations. With appropriate consideration of materials' compatibility, single-use disposable equipment (supplied sterile when necessary) may be utilized.

EQUIPMENT HAVING INDIRECT CONTACT WITH COMPONENTS, CONTAINERS, CLOSURES, AND STERILE PRODUCTS

Equipment having a significant impact on product quality, that does not contact components, primary packaging materials, and sterile products, includes the electro-mechanical elements (non-product contact) of filling machines, stoppering machines, and sterilizers. The performance of this equipment can change fill weight, particle size, moisture level, content uniformity, container–closure integrity, and other essential quality attributes. This equipment is ordinarily located near exposed product contact equipment surfaces. For example, a pre-assembled filling set (product contact equipment) may be installed on a filling machine (significant impact without product contact), which provides control over the fill volume or weight. The surfaces of this equipment must be compatible with the cleaning and microbial decontamination and/or sterilization agents employed.

OTHER EQUIPMENT

Some equipment has only an indirect impact on product quality, for example, conveyors, turntables, balances, air samplers, and carts. The influence of this equipment is largely on the environments in which the product is made. The exposed surfaces of this equipment must be compatible with the cleaning and decontamination agents used.

Facilities

Sterile manufacturing operations are supported by administrative, laboratory, maintenance, and warehouse functions and other activities. The impact of these operations on the location and overall design of the sterile manufacturing area must be considered. Emphasis should be given in facility design to the flows of materials, components, personnel, equipment, and waste streams throughout the facility and to the orderly transition of items between environments of different classifications to prevent mix-up and avoid product contamination. Facility environmental and utility systems must be designed to minimize microbial, chemical, and particulate contamination. The facility design must be supported by practices and procedures such as cleaning and decontamination, gowning, and material transfer. The architectural details of the facility infrastructure must consider the

[2] Sterilization out-of-place would be used on a less frequent basis.

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means for cleaning and disinfection. Detailed design recommendations can be found in the International Society for Pharmaceutical Engineering's *Baseline Guide: Sterile Product Manufacturing Facilities* (*7*).

The core activities for sterile product manufacture are carried out in classified environments operating in conformance with the ISO 14644 series of standards (*8*). A pressure cascade descending from the more critical areas to less critical is commonplace. In general, the more protection that materials have from potential sources of contamination during holding or processing, the less impact the facility has on the process outcome. Human operators within ISO 14644 classified cleanrooms used in aseptic processing are the greatest risk to product safety; therefore, no single risk mitigation factor in aseptic processing is more important than minimizing risk emanating from gowned operators.

Early-stage container–closure and equipment washing and preparation are carried out in lower classification areas (ISO 7–8). Nonsterile formulation is typically carried out in ISO 6–7 environments. The production materials are introduced into the processing area where subsequent steps are performed. The bioburden level of the materials influences the detailed design of the facility and its controls. Nonsterile materials (e.g., formulation, containers, closures, equipment, and utensils) require subsequent sterilization and, where necessary, depyrogenation. Sterile materials are introduced through airlocks and pass-throughs. The facility design controls for the background, and processing environments should be chosen to preserve the intended microbial attributes of the in-process and finished materials.

Table 1 provides some examples of formulation and filling environments.

Table 1. Examples of Environments for Processing

Processing Technology	Background Environment	Processing Environment	Reference
Conventional cleanroom	ISO 5–7	ISO 5	FDA AP 2004 (*9*), EMA Annex (*10*)
Closed RABS	ISO 7	ISO 5	ISPE (*11*), PHSS (*12*)
Open RABS	ISO 5	ISO 5	ISPE (*11*), PHSS (*12*)
Closed isolators	CNC[a]	ISO 5	FDA AP 2004 (*9*), PDA TR No. 34 (*13*), ISPE (*7*)
Open isolators	CNC-ISO 8	ISO 5	FDA AP 2004 (*9*), PIC/S (*14*), PDA TR No. 34 (*13*), ISPE (*7*)
Blow-fill-seal/form-fill-seal	ISO 5–7	ISO 5	FDA AP 2004 (*9*), Baseman (*15*)
Closed systems	CNC	Not applicable[b]	PDA TR No. 28 (*5*); Agalloco, Hussong, et al. (*16*)
Terminal sterilization	ISO 8	ISO 5–7	EMA Annex (*10*)

[a] Controlled non-classified—a non-classified controlled environment with filtered air supply.
[b] As the process occurs with a closed system, there is no separate processing environment.

[NOTE—Table values represent the operational condition and are adapted from the reference documents.]

CONVENTIONAL CLEANROOM

The critical activities are performed in ISO 5 environments supported by surrounding ISO 5–7 environments where gowned personnel are normally located. There may be only limited separation between gowned personnel and sterile materials and product contact surfaces. The critical activities are performed within a unidirectional airflow environment. Decontamination of the cleanroom is commonly performed by personnel.

RESTRICTED ACCESS BARRIER SYSTEMS

The typical RABS provides ISO 5 unidirectional air within the barrier and is situated in a conventional ISO 5–7 cleanroom. RABS may be designed to allow for opening of barriers to enable human intervention, or they may be designed to operate closed with the same operational restrictions regarding operator access that applies to isolators (see below). Air overspill from within the barrier is designed to prevent the ingress of contamination. Operator manipulation of sterile items is achieved using glove ports, and material transfers are accomplished without opening the system. A RABS that is opened mid-process should be treated as a conventional cleanroom (see above). RABSs require decontamination prior to use. This may be accomplished either manually or using automated systems.

ISOLATORS

Isolators provide complete separation between personnel and the enclosed ISO 5 processing environment. A defined pressure differential is maintained between the ISO 5 environment and the surrounding area. Air overspill provides an aerodynamic seal at points where the product exits the isolator into an external environment of lesser classification or no classification. The use of unidirectional air is not required in isolators. Isolators are commonly decontaminated using automated systems.

BLOW-FILL-SEAL AND FORM-FILL-SEAL

These technologies form, fill, and seal flexible walled containers in an ISO 5 environment. Blow-fill-seal (BFS) and form-fill-seal (FFS) equipment configurations allow for installation in a variety of background environments. The critical activities are

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performed within a unidirectional airflow environment. Decontamination is performed as is common for the background environment.

CLOSED SYSTEMS

These systems provide for complete separation of production materials from personnel and surrounding environment. Closed systems can be single- or multiple-use vessels/chambers with means for materials ingress/egress. The designs avoid any human interaction or environmental contact with sterile materials. These systems vary in complexity and are sterilized either in situ or prior to use.

TERMINAL STERILIZATION

Filling systems and environments for containers to be terminally sterilized can be accomplished in ISO 7 or better environments. The critical activities are performed within a unidirectional airflow environment. Decontamination of the cleanroom is commonly performed by personnel.

Materials (Active Pharmaceutical Ingredients, Excipients, and Process Aids)

The preparation of sterile products encompass a wide range of materials including active pharmaceutical ingredients (small and large molecules), excipients, solvents (usually water), process gases and processing aids, all of which contribute to the microbiological quality attributes of the product. Depending upon the product being manufactured, this can require consideration of bacterial, endotoxin, and particulate contamination. Specific microbiological quality testing requirements for inactive and active ingredients testing is often specified in a relevant *USP–NF* monograph. Requirements for microbiological testing for total aerobic bacteria, yeast and mold counts, and specified organisms are given in *Microbial Enumeration Tests* ⟨61⟩ and *Tests For Specified Microorganisms* ⟨62⟩, and the recommended but non-mandatory enumeration targets for microbiological testing are given in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩. Some specific products may require other testing for specified organisms, including viruses, as a requirement of their regulatory approval. Requirements for bacterial endotoxin testing in injectable products are covered in ⟨85⟩, which describes qualification of *Limulus* amebocyte lysate testing for acceptable levels of bacterial endotoxin. Raw material specifications must be appropriate to ensure that the manufacturing process consistently results, as demonstrated through process validation, in products conforming to the microbiological critical quality attributes.

Microbial contamination may be present on/in active pharmaceutical ingredients, excipients, and primary packaging materials. Controlling bioburden in materials and formulated product is a critical aspect of sterility assurance. The nature of the active pharmaceutical ingredient, the excipients, and the compounded product intended to be sterile are critical elements of the product knowledge. Recognizing the potential impact on critical quality attributes, the microbiological attributes of materials should be controlled through adequate supplier controls, shipping, receipt, sampling, handling, and storage. These controls should be commensurate with the microbiological risks to process controls and sterile product safety. Laboratory results should not be utilized to rationalize inadequate process controls on the part of the supplier.

The potential impact of the materials on the microbiological critical quality attributes of presterilized and finished product must be assessed by evaluating them with appropriate compendial assays or validated alternatives. Risk assessment should consider the origin of the material (e.g., fermentation, chemical synthesis, biologically derived, enzymatic, semi-synthetic synthesis, natural origin). Materials of biological origin may have higher inherent microbiological risk than materials derived solely from chemical synthesis. Materials may have inherent physicochemical properties that mitigate microbiological risks (e.g., low water activity, extreme pH, inherent antimicrobial properties) or increase microbiological risks (e.g., aqueous solution, growth supportive nutrients); this risk of supporting microbial proliferation must be assessed.

Monitoring

Environmental monitoring is employed to qualitatively assess the effectiveness of the design and operational controls to provide suitable facility hygiene. It is neither a substitute for good facility, equipment, and process design, nor compensation for deficient practices and behavior. There are inherent limitations with all forms of viable and non-viable monitoring in terms of sample size, sample location, and recovery capability that preclude their use as anything more than an indication that a facility is operating within an acceptable state of control. Monitoring provides only a snapshot in time of the actual environmental conditions and excessive sampling due to its potentially intrusive nature can actually impair product safety or generate counts unrelated to process performance by increasing activity proximate to the critical zone.

Independent of air, surface, and personnel monitoring, media fills and sterility testing provide additional (albeit analytically and statistically limited) means to evaluate the robustness of the cleanroom design, performance, and effectiveness of cleaning/decontamination procedures, personnel gowning integrity, and aseptic practices.

Environmental control can be measured only by the monitoring performed. Satisfactory monitoring performance is the result of proper design and operation as described in this chapter and not a means to establish that condition. Performance criteria are established according to the classification of the room and its usage (see ⟨1116⟩).

VIABLE MONITORING

Viable monitoring consists of detecting and estimating the level of culturable microorganisms in the air, on surfaces, and on personnel. Sampling locations are defined following a risk assessment and sampling is executed by trained operators using a variety of methods including:

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- Active air sampling
- Passive air sampling
- Viable particle counting using fluorescence technology
- Contact-plate sampling of surfaces, gloves, and gowns
- Swabbing of surfaces
- Personnel monitoring

NON-VIABLE MONITORING

Non-viable monitoring measures the number and size of particulates present in the air. It can be used to initially classify the cleanroom in accordance with ISO 14644-1 and to assess routine manufacturing conditions (*8*). When used for the purposes of monitoring, it can be performed under static conditions (no activity) and/or dynamic conditions (routine operation). Non-viable particle monitoring is performed using calibrated particle counters.

MEDIA FILLS/ASEPTIC PROCESS SIMULATIONS

Process simulations are exercises in which the performance of an aseptic activity is evaluated using a sterile growth medium. The medium can be directly substituted for the product or added to it. Aseptic process simulations are typically performed before the introduction of new or revised process components (e.g., products, facilities, equipment, personnel, containers and closures, and processes) and periodically thereafter (*17*). Process simulations should be fully representative of processing conditions and activities utilized during routine production.

STERILITY TESTING

The sterility test is a harmonized compendial test. It must be understood that while execution of the test is required for the release of sterile products where parametric release has not been approved, it cannot prove the sterility of the materials tested. It should be recognized that parametric release is the default mode of sterile product release.

Personnel

Personnel play an essential role in the preparation of sterile products. The essential activities they perform include cleaning, assembly, equipment operation, material transfer, environmental monitoring, and decontamination. While personnel are often necessary for the performance of these activities, the contamination derived from them must be prevented from entering the production materials before and after sterilization. The importance of the controls necessary to minimize exposure to and the release of human microbial contaminants in a sterile product manufacturing environment cannot be overstated.

The personnel involved in the preparation of sterile products must:

- Understand the principles of microbiology, sterilization/depyrogenation, aseptic processing, and contamination control
- Be proficient and diligent in gowning practices. Personnel required to wear aseptic gowning should periodically demonstrate their ability to properly gown
- Adhere to proper aseptic technique during all aseptic activities even when these are performed in a RABS or isolator. Periodic demonstration of these skills can be beneficial
- Be familiar with and adhere to standard operating procedures
- Practice good personal hygiene to minimize contamination potential
- Be trained in the proper and safe operation of necessary equipment
- Be monitored microbiologically after performing aseptic operations

Procedures

Written procedures define the operations that have been determined through validation studies and experience to be effective in controlling and facilitating the manufacture and quality of pharmaceuticals and biopharmaceuticals. Procedures are especially important for the critical processes designed to assure the sterility of terminally sterilized and aseptically produced drug products. Procedures should be periodically reviewed and evaluated to ensure they are effective and current.

INTERVENTIONS AND INTERVENTION PROCEDURES

There are two types of interventions associated with the aseptic production of sterile drug products. Inherent interventions are those activities that are an integral part of the aseptic process and are performed during the production of every batch. They include set-up, replenishment of components, weight and volume checks and adjustments, and environmental monitoring. Corrective interventions are those activities that correct problems and might not be performed during the production of every batch. They can be minimized and should be avoided through careful process design. Examples include stopper jams, broken and fallen glass, defective container seals, liquid leaks, and mechanical failures requiring manual correction.

Each intervention, whether inherent or corrective, should be covered by written procedures sufficiently detailed to enable personnel to perform the intervention correctly, and to perform the intervention the same way each time regardless of whomever performs it. For example, procedures should specify the number of units, their locations, and how the units are to be removed, and personnel must be trained so they can correctly execute the procedures. No intervention should be permitted

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for which there is not a defined procedure. The following concepts should be considered during development, review, and implementation of intervention procedures.

1. Interventions performed during all forms of processing must be recognized as increasing the risk of contamination dissemination and are to be avoided or designed out of the process to the extent possible
2. Procedures for interventions should be critically reviewed to eliminate and/or simplify aseptic processes by reducing the frequency of inherent interventions and making all interventions easier to perform
3. Interventions should be designed for minimal risk of contaminating sterile and nonsterile materials
4. All interventions should be performed using sterilized tools whenever possible
5. Intervention procedures should be established in detail for all inherent interventions, and more broadly for corrective interventions (where some flexibility is necessary due to greater diversity)
6. Interventions should be incorporated in periodic media fills to evaluate the aseptic practices of the operators

Operators should initially, and periodically thereafter, be trained in all of the procedures they are expected to perform. Considerations for operator activity during the non-aseptic filling of containers should parallel those described above to minimize the potential for contamination ingress with somewhat less rigor than those needed for aseptic operations.

Sterilization

The most effective means for the control of microbial population is sterilization, a process that either kills or removes viable microorganisms. In the production of sterile products, sterilization processes are used to prevent microbial contamination. Terminal sterilization processes that reproducibly destroy microorganisms in the final product container are the preferred means for the production of sterile products. Sterile products that cannot be terminally sterilized rely on individual sterilization processes (e.g., steam, radiation gas, filtration) for the various materials that comprise an aseptically processed sterile product. In addition, sterilization processes are used for product contact and other non-product contact items used in a variety of applications during the preparation of sterile products to provide absolute control of bioburden. Details on sterilization processes are provided in ⟨1229⟩.

Utilities

The manufacture of sterile products requires utilities that can have a substantial impact on the final product. Some of the utilities in the facility can become an integral part of the formulated product (e.g., *Water for Injection*, *Nitrogen*) and appropriate design of the production and distribution system for these is essential. The systems for these are tightly controlled and frequent monitoring of the utilities produced is customary. These utilities may be also used in the process, and not become a part of the sterile product. Other utilities (e.g., clean steam, compressed air, *Purified Water*) that are used in the cleaning/decontamination of facilities, and/or preparation of equipment, containers, and closures can also be subjected to microbial control.

Utilities included in the product, in direct product contact, and in the preparation of equipment, containers, closures, and other items must meet the requirements defined in the appropriate *USP–NF* monograph. The systems for their preparation should be subject to formalized controls that maintain a controlled state over time. This is accomplished through a number of related practices essential for continued use of the system over an extended time. The essential practices to maintain controlled status of the utility systems include: calibration, change control, corrective and preventive maintenance, and ongoing process control.

There are other less impactful utilities (e.g., vacuum, cooling water) necessary for the operation of the facility and equipment. Although these non-product contact utilities may lack monograph requirements, their reliable operation is necessary for consistent production of sterile products.

SUMMARY

The safety of products labeled sterile requires that their critical quality attributes consistently meet specifications. Sterility is the most essential quality attribute. Sterility is an unqualified concept in which an item is devoid of living microorganisms capable of reproduction. Monitoring of all types, environmental (viable and non-viable; air, surface, and personnel), media fills, and sterility tests are forms of microbiological analysis that have been historically employed as proof of "sterility". These assessment tools cannot provide definitive evidence of either "sterility" or "nonsterility", because the means to confirm either of those conditions non-destructively is not scientifically possible. Confidence in sterile product manufacturing is realizable only by a holistic approach in which all of the supportive elements of the operations are given due consideration and emphasis (*17*).

The absolute nature of sterility presents the practitioner with an inherent paradox—there is no ready means to demonstrate sterility of an item in the absolute sense regardless of the means used to provide it. Test methods including those defined in this compendium (*Sterility Tests* ⟨71⟩) utilize a number of samples taken from a large population to infer the "sterility" of the whole. Sterilization procedures including those validated for parametric release can deliver a low probability of a nonsterile unit, but not absolute assurance that "sterility" actually exists.

The uncertainty associated with proof of "sterility" notwithstanding, the means by which sterility assurance is provided are reasonably well defined. The "sterility" of any item is definitively established by the process controls summarized in this chapter rather than any form of monitoring or sampling. For terminally sterilized products, greater weight can be placed on the sterilization process utilized than on any form of testing. Confidence in aseptic processing is a result of sound design, reliable equipment, quality materials, effective procedures (including supportive sterilization processes), and personnel proficiency rather than through sampling dependent sterility testing, microbiological monitoring, and process simulation.

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▲ 2S (USP41)

⟨1216⟩ TABLET FRIABILITY

This general information chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. The harmonized texts of these three pharmacopeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present *United States Pharmacopeia* general information chapter method. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablet friability supplements other physical strength measurements, such as tablet breaking force.

Use a drum,* with an internal diameter between 283 and 291 mm and a depth between 36 and 40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see figure for a typical apparatus). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ±1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

*The apparatus meeting these specifications is available from laboratory supply houses such as VanKel Technology Group, 13000 Weston Parkway, Cary, NC 27513, or from Erweka Instruments, Inc., 56 Quirk Road, Milford, CT 06460.

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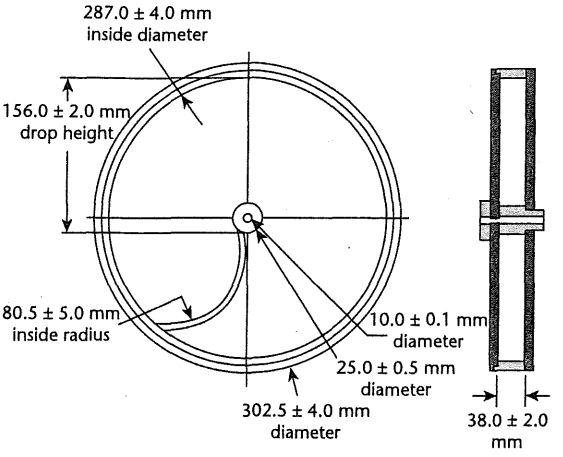

Tablet Friability Apparatus

For tablets with a unit weight equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit weight of more than 650 mg, take a sample of 10 whole tablets. The tablets should be carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the weight loss is greater than the targeted value, the test should be repeated twice and the mean of the three tests determined. A maximum mean weight loss from the three samples of not more than 1.0% is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

Drums with dual scooping projections, or an apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

⟨1217⟩ TABLET BREAKING FORCE

Change to read:

INTRODUCTION

There are a variety of presentations for tablets as delivery systems for pharmaceutical agents, such as rapidly disintegrating, slowly disintegrating, eroding, ▲and▲ (USP 1-May-2019) chewable. ▲The concepts of this chapter are also applicable to lozenges.▲ (USP 1-May-2019) Each of these presentations places a certain demand on the bonding, structure, and integrity of the compressed matrix. Tablets must be able to withstand the rigors of handling and transportation experienced in the manufacturing plant, in the drug distribution system, and in the field at the hands of the end users (patients/consumers). Manufacturing processes such as coating, packaging, and printing can involve considerable stresses, which the tablets must be able to withstand. ▲For these reasons, several tests are available to assess the strength of the compact as well as the response to contact with water and other liquids. These tests include *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Tablet Friability* ⟨1216⟩, and *Tablet Breaking Force*, the subject of this chapter. The mechanical strength of tablets is of considerable importance and is routinely measured. Tablet strength serves both as a criterion by which to guide product development and as a quality control specification. An acceptable value for the strength of the compact must consider the intended use as well as the mechanism of release of the active ingredient(s) from the dosage form.▲ (USP 1-May-2019)

One commonly employed test of the ability of tablets to withstand mechanical stresses determines their resistance to chipping and surface abrasion by tumbling them in a rotating cylinder. The percentage weight loss after tumbling is referred to as the friability of the tablets. Standardized methods and equipment for testing friability have been provided in ⟨1216⟩. ▲In addition to the loss of active drug, chipping and abrasion can each have a significant impact upon the success of subsequent manufacturing operations such as coating and packaging, and impact the consumer's perceived elegance of the dosage form.▲ (USP 1-May-2019)

Another measure of the mechanical integrity of tablets is their "breaking force", which is the force required to ▲produce failure (i.e., breakage)▲ (USP 1-May-2019) in a specific plane. The tablets are generally placed between two platens, one of which moves to apply sufficient force to the tablet to cause fracture. For conventional, round (circular cross-section) tablets, loading occurs across their diameter (sometimes referred to as diametral loading), and fracture occurs in that plane. ▲As previously stated, friability and breaking force measure different aspects of the compact strength. Depending upon the formulation, manufacturing method, and intended use, the results from one of the tests may be a better indicator of the quality of the dosage

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form than the other. This determination is best made at the time the formulation and manufacturing process are defined. ▲ (USP 1-May-2019)

The breaking force of tablets is commonly called "hardness" in the pharmaceutical literature; however, the use of this term is misleading. In material science, the term "hardness" refers to the resistance of a surface to penetration or indentation by a small probe. The term "crushing strength" is also frequently used to describe the resistance of tablets to the application of a compressive load. Although this term describes the true nature of the test more accurately than does "hardness", it implies that tablets are actually crushed during the test, which often is not the case ▲nor the intent of the breaking force determination. ▲ (USP 1-May-2019) Moreover, the term "strength" in this application can be questioned, because in the physical sciences that term is often used to describe a stress (e.g., tensile strength). Thus, the term "breaking force" is preferred and will be used in the present discussion.

▲During manufacture of compressed tablets, decisions must be made regarding tablet weight, thickness, friability, and breaking strength targets. Control of tablet weights must be given priority because weight control directly correlates with dosing accuracy. Once the desired weight is achieved, decisions must be made concerning the relative importance of the thickness, loss on drying, friability, and breaking strength. While thickness may be viewed as a physical parameter that only influences the appearance of the tablet, it also determines the pore volume of the compact (i.e., solid fraction, see also *Tablet Compression Characterization* ⟨1062⟩). While thickness, friability, and breaking strength are related, the relationships are not clear or easily predicted. As illustrated in ⟨1062⟩, the thickness of the tablet at constant weight may not be varied without influencing the breaking strength. It is therefore important for the manufacturer to identify which parameter most closely correlates with desired product performance. ▲ (USP 1-May-2019)

Change to read:

TABLET BREAKING FORCE DETERMINATIONS

Early measuring devices were typically hand operated. For example, the Monsanto (or Stokes) hardness tester was based on compressing tablets between two jaws via a spring gauge and screw. In the Pfizer hardness tester, the vertically mounted tablet was squeezed in a device that resembled a pair of pliers. In the Strong-Cobb hardness tester, the breaking load was applied through the action of a small hydraulic pump that was first operated manually but was later motorized. Problems associated with these devices were related to operator variability in rates of loading and difficulties in proper setup and calibration. Modern testers employ mechanical drives, strain gauge-based load cells for force measurements, and electronic signal processing, and therefore are preferred. However, several important issues must be considered when using them for the analytical determination of breaking force; these are discussed below.

Platens

The platens should be parallel. Their faces should be polished smooth and precision-ground perpendicularly to the direction of movement. ▲In addition, the face of the platen should be perpendicular to the surface on which the tablet is placed to ensure the platen uniformly contacts the side of the tablet from top and bottom. ▲ (USP 1-May-2019) Perpendicularity must be preserved during platen movement, and the mechanism should be free of any bending or torsion displacements as the load is applied. The contact faces must be larger than the area of contact with the tablet. ▲These considerations ensure that the applied load is operating over a consistent surface area of the dosage form. ▲ (USP 1-May-2019)

Rate and Uniformity of Loading

Either the rate of platen movement or the rate at which the compressive force is applied (i.e., the loading rate) should be constant. Maintaining a constant loading rate avoids the rapid buildup of compressive loads, which may lead to uncontrolled crushing or shear failure and greater variability in the measured breaking force. However, constant loading rate measurements may be too slow for real time monitoring of tablet production.

The rate at which the compressive load is applied can significantly affect results, because time-dependent processes may be involved in tablet failure (*1*). How a tablet matrix responds to differences in the loading rate depends on the mechanism of failure.

▲Additional discussion of the consolidation mechanism of materials is provided in ⟨1062⟩. Since different formulations of tablets exhibit varying amounts of strain rate sensitivity, it is important to conduct the breaking force test in a controlled manner and recognize that instruments that operate with different platen movement or loading rates may not give comparable results for the same product. ▲ (USP 1-May-2019)

The test must be run consistently with equipment that has been routinely calibrated. Changing from testing units of different designs or from different manufacturers will require comparison of data to ensure that the two units are subjecting the dosage form to similar stress in a similar manner. Currently available equipment provides a constant loading rate of 20 newtons (N) or less per second or a constant platen movement of 3.5 mm or less per second. Controlled and consistent breaking is an important test procedure attribute. To ensure comparability of results, testing must occur under identical conditions of loading rate or platen movement rate. Since there are certain advantages to each system of load application, both are found in practice. Because the particular testing situation and the type of tablet matrix being evaluated will pose different constraints, there is also no basis to declare an absolute preference for one system over the other. This general chapter proposes consideration of both approaches.

The different methods may lead to numerically different results for a particular tablet sample, requiring that the rate of load application or displacement must be specified along with the determined breaking force.

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Dependence of Breaking Force on Tablet Geometry and Mass

Measurements of breaking force do not take into account the dimensions or shape of the tablet. ▲▲ (USP 1-May-2019) Tablet orientation and failure should occur in a manner consistent with ▲the orientation used and failure observed▲ (USP 1-May-2019) during the development of the dosage form. For direct comparisons (i.e., without any normalizations of the data), breaking force measurements should be performed on tablets having the same dimensions, ▲weights,▲ (USP 1-May-2019) geometry, and consistent orientation in test equipment.

Tablet Orientation

Tablet orientation in diametral compression of round tablets without any scoring is unequivocal. That is, the tablet is placed between the platens so that compression occurs across a diameter. However, tablets with a unique or complex shape may have no obvious orientation for breaking force determination.

▲Because the breaking force for unembossed, noncircular cross-section tablets will likely depend on the tablet's orientation in the tester, it is best to settle on a standard orientation, preferably one that provides a consistent failure plane and that is readily reproduced by operators, to ensure comparability of results.▲ (USP 1-May-2019) In general, tablets are tested either across the diameter or parallel to the longest axis. Scored tablets have two orientation possibilities. When they are oriented with their scores perpendicular to the platen faces, the likelihood that tensile failure will occur along the scored line increases. This provides information about the strength of the matrix at the weakest point in the structure. When scored tablets are oriented with their scores parallel to the platen faces, more general information about the strength of the matrix is derived.

Capsule-shaped tablets or scored tablets may best be broken in a three-point flexure test (*2*). A fitting, which is either installed on the platens or substituted for the platens, supports the tablet at its ends and permits the breaking load to be applied to the opposite face at the unsupported midpoint of the tablet. The fittings are often available from the same source that supplies the hardness tester.

▲Since the orientation of the dosage form critically impacts the result of the test, it must be clearly defined in the test procedure and produce a consistent failure plane in the dosage form for the results to be comparable. The plane of failure should always be noted. If, while maintaining orientation, the plane of failure differs for a batch or sub-batch of tablets relative to previous results and direct comparison of the numerical value is not possible, this observation in itself can be an important indicator of a compression problem.▲ (USP 1-May-2019)

Units, Resolution, and Calibration

Modern breaking force testers are usually calibrated in kiloponds or newtons. The relationship between these units of force (*3*) is 1 kilopond (kp) = 1 kilogram-force (kgf) = 9.80 newtons (N). The test results should be expressed in standard units of force which facilitate communication. Some breaking force testers also will provide a scale in Strong-Cobb units (SCU), a carryover from the days when Strong-Cobb hardness testers were in common usage. The conversion between SCU and N or kp must be viewed with caution, because the SCU is derived from a hydraulic device and is a pressure.

Generally, contemporary breaking force testers use modern electronic designs with digital readouts. Some units also have an integral printer or may be interfaced with a printer. Breaking forces should be readable to within 1 N.

Breaking force testers should be calibrated periodically. The force sensor as well as the mechanics of the apparatus needs to be considered. For the force sensor, the complete measuring range (or, at a minimum, the range used for measuring the test sample) should be calibrated to a precision of 1 N, using either the static or dynamic method. Static calibration generally employs traceable counterweights; at least three different points are checked to assess linearity. Dynamic calibration makes use of a traceable reference-load cell that is compressed between the platens. The functional calibration of a breaking force test apparatus should also confirm that the velocity and the constancy of velocity for load application or displacement are within prescribed tolerances throughout the range of platen movement.

Sample Size

▲Interpretation of the breaking force data must consider not only the mean value but the consistency of the test results for multiple tablets. The range of breaking force values may provide valuable information on the consistency of the tableting process. Excessive variability in values may reflect issues with die filling, weight control, or orientation of the dosage form in the test equipment. Additionally, the breaking strength result will reflect the orientation of the failure plane. For results to be compared, the tablets must fail in the same manner throughout the test. In cases where breaking force may be particularly critical, the average plus individual breaking force values should be reported.▲ (USP 1-May-2019)

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TENSILE STRENGTH

▲Tensile strength is an intensive materials property, which by definition does not depend on the size or shape of the material being measured. The measurement of tensile strength provides a more fundamental measure of the mechanical strength of the compacted material and takes into account the geometry of the tablet. The application of a vertical force (F), across the diameter of a disc-shaped tablet generates a horizontal stress (σ_x), which is tensile and constant along the centerline of the tablet. The value of this stress is given by the following equation, which applies to diametral compression of cylindrical tablets:

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$$\sigma_X = 2F/\pi DH$$

σ_X = horizontal stress
F = breaking force
D = tablet diameter
H = tablet thickness

The derivation of this equation may be found in standard texts (*4–6*); it is based on elastic theory and the following assumptions:

1. The tablet is an isotropic body
2. Hooke's law is obeyed
3. The modulus of elasticity in compression and in tension is the same
4. Ideal point loading occurs

If the force is increased to the point of tablet fracture and if the tablet breaks into two roughly equal halves, the corresponding tensile stress at failure is historically reported as the "tensile strength". However, recent studies (*7*) show that the tensile strength calculated using this method can be half or less of the actual tensile strength as measured in a three-point bend test. The discrepancy occurs because tablet fracture initiates under the intense shear stresses (*8*) that are present near the point of contact between the tablet and the compression platen. The intense shear stresses allow cracks to form under Mode III fracture conditions (out-of-plane shearing), even though σ_X is still below the strength of the compact. Once fracture initiates, the cracks propagate along the center line by Mode I fracture (opening) and at tensile stresses well below the true tensile strength of a crack-free tablet. Despite these errors the diametrical strength is still useful in many applications (*9*). Moreover, the data will be normalized with respect to tablet dimensions, because both diameter and thickness are included in the equation.

The derivation has been extended to convex-faced tablets (*10,11*):

$$\sigma_X = (10F/\pi D^2) \times [(2.84H/D) - (0.126H/W) + (3.15W/D) + 0.01]^{-1}$$

σ_X = tensile stress
F = breaking force
D = tablet diameter
H = tablet thickness
W = central cylinder thickness (tablet wall height)

Other examples may be available in the literature (*12*).▲ (USP 1-May-2019)

Bending or flexure of tablets is another option for determining the tensile strength of tablets. Under ideal loading conditions, a breaking load applied to the unsupported midpoint of one face will result in the generation of pure tensile stress in the opposite face. If the tablets are right circular cylinders and are subjected to three-point flexure, the tensile strength may be estimated using the following equation (▲*13*▲ (USP 1-May-2019)):

$$\sigma_X = 3FL/2H^2D$$

σ_X = tensile stress
F = breaking force
L = distance between supports
H = tablet thickness
D = tablet diameter

▲The value of L should be chosen judiciously to minimize errors in this calculation (*9*).▲ (USP 1-May-2019) The assumptions are the same as those for calculating tensile strength from diametral compression. However, tensile strengths determined by flexure and diametral compression may not agree because of likely nonideal loading and the induction of shear failure during testing.

Change to read:

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⟨1222⟩ TERMINALLY STERILIZED PHARMACEUTICAL PRODUCTS—PARAMETRIC RELEASE

Change to read:

▲INTRODUCTION

Parametric release is a practice to release finished product that relies on process control in lieu of end product testing to establish that a product is safe, pure, efficacious, and of suitable strength for commercial or clinical use. Parametric release is based on demonstrating that in-process conditions relevant to the establishment of key product quality attributes were attained and maintained throughout the relevant manufacturing steps. One attribute for which in-process controls would replace end product testing is sterility. The radiation sterilization of articles described in *Radiation Sterilization* ⟨1229.10⟩ performed in accordance with International Organization for Standardization (ISO) 11137 was the first sterilization process to use in-process controls and monitoring to replace sterility testing. The application of parametric release specifically to the attribute of sterility for commercial use requires prior regulatory approval.

Appropriately designed, validated, and controlled sterile product manufacturing systems are capable of exceptionally consistent performance in the preparation of products that have a probability of a nonsterile unit (PNSU) of $\leq 10^{-6}$. The exceptionally low probability of microbial presence in products manufactured using these systems renders the analytical methods described in *Sterility Tests* ⟨71⟩ statistically ineffectual.

While theoretically the limit of detection of the sterility tests is one viable cell, in actuality the limit of detection is likely far greater. A major limitation of ⟨71⟩ is that it is based on a limited sample. The probability of failing a sterility test given a contamination rate of 0.1% (an unacceptably high level of contamination) is 2% (where $n = 20$). For the contamination control capabilities achieved by well-controlled manufacturing systems, the probability of a sterility test detecting that level of contamination would likely be well below the limit of detection of the procedure. For these reasons, parametric release is the default mode of product release and should be used in lieu of sterility testing unless parametric release is not feasible.

In a parametric release program, sterility assurance is achieved by process, facility, and systems engineering; the establishment of appropriate risk-based user requirement specifications; and is conclusively demonstrated by the establishment, control, and monitoring of process parameters that confirm those user requirements are met. All modalities of sterile manufacturing should achieve a probability of a PNSU of $\leq 10^{-6}$. Parametric programs of product release should ensure all elements referenced in *Sterilization Assurance* ⟨1211⟩ consistently support the highest level of control.

Conditionally, terminal sterilization processes for the manufacture of products labeled sterile are candidates for parametric release. Terminal sterilization is the application of a suitable sterilization technology to a product, in its final sealed container or packaging, in a manner that achieves a probability of a PNSU of $\leq 10^{-6}$. Terminal sterilization may be conducted using any sterilization method that can be demonstrated to achieve a PNSU of $\leq 10^{-6}$ while at the same time retaining all other required product quality attributes. Examples of sterilization methods that have been successfully used to sterilize drug products or medical devices in their primary package are moist heat, dry heat, gas, and radiation. Processing parameters, critical processing parameters, and operational ranges ensuring the requisite microbial destruction should be understood, defined, measured, and controlled to support parametric release.

USER REQUIREMENTS

Establishing precise, clearly defined requirements that demonstrate in-process conditions are attained and maintained throughout the relevant manufacturing steps is essential for parametric release. Therefore, organizations wishing to develop and validate a candidate parametric release program must define the necessary user requirements specification (URS) and ensure that all conditions within that specification are met. Process control conditions and validation within the URS should be acceptable to relevant competent regulatory authorities.

The URS should include all critical functions of the technology, equipment, manufacturing process, environmental requirements, operational requirements, and other important characteristics necessary for ensuring provision of a sterile product. Since these requirements will be specific to the organization, the sterilization process utilized, the product and its intended use, the URS should be generated by the user.

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In developing and validating a parametric release program, critical quality attributes and process parameters should be included in the URS:

Critical quality attributes: Criteria immediately before and after sterilization should be achieved to ensure that the sterility of every unit is sustained through expiry.

Process parameters: These should be accurate predictors of the assurance of product sterility. The operating ranges are developed based on the sterilization process, process capability, calibration tolerance limits, and process parameter criticality. Process parameters necessarily should be based upon a thorough process knowledge and understanding of risk. Control of critical process parameters within their validated operating ranges is necessary to assure the sterility of product manufactured within a parametric release program. Any failure to adequately control critical process parameters within the established, validated operating ranges will result in the disposal of the processed product.

Presterilization critical quality attributes and process parameters: Presterilization process parameters are important to ensure a consistent and minimized microbiological challenge to the sterilization process. For a parametric release program, the level of control of the environment and presterilization product intermediate bioburden (amount, frequency of recovery, and species) should be within validated ranges and ensure the efficacy of the subsequent sterilization. Recovery of species or forms of microorganisms that are generally recognized as representing an increased challenge to the sterilization process (e.g., spores for moist heat sterilization) should be infrequent as demonstrable by historical data. The sterilization process might not effectively control pyroburden (which is usually endotoxins). Therefore, pyroburden should be appropriately controlled throughout the manufacturing process. *Table 1* summarizes the minimum presterilization quality attributes and associated process parameters.

Table 1. Presterilization Critical Quality Attributes and Associated Process Parameters

Critical Quality Attributes	Process Parameters
Bioburden and the absence of specific microorganisms exhibiting an increased challenge to the mode of sterilization	• Pre-container-filling filter integrity[a] • Hold times (formulated product and filled product units)[a] • Cleaning and sterilization (where appropriate) of product contact surfaces[a] • Environmental and personnel control performance • Bioburden of excipient, raw material, drug substance, container, closure
Pyroburden	• Hold times (formulated product and filled product units)[a] • Cleaning and sterilization (where appropriate) of product contact surfaces[a] • Pyroburden of excipient, raw material, drug substance, container, closure
Filled container and closure integrity	• Filling and sealing control performance[a] • Container and closure component dimensions and material properties

[a] Critical process parameters.

Sterilization process parameters: Control of process parameters during the sterilization process is important to ensure a consistent and predictable level of product sterilization within the process' validated state. During the sterilization process, critical process parameters are monitored and recorded. Appropriate chemical, physical, or biological load monitors should be used to demonstrate lethality imparted upon the load. For example, the parametric release of most heat terminally sterilized products should use Class 5 indicators or, in certain circumstances, Class 3 (ISO 15882:2008) load monitors. Load monitors are located at specific locations in the load determined from development and qualification data to ensure each unit receives the required minimum sterilizing conditions. Certain container–closure configurations may possess sites that are less accessible to the physical or chemical agent used to terminally sterilize (e.g., the obscured interface between a container and its closure). In these circumstances, biological indicators and heat penetration probes (where applicable) should be placed at such locations during development and qualification studies to determine the required process parameters. For a parametric release program, the risk of human error should be minimized by use of electronic and automated systems to ensure the control of the sterilization cycle. In addition to qualified personnel, an automated electronic assessment of the sterilization cycle should assess the critical process parameters against the validated values. The assessment should be recorded and evaluated for the disposition of product. The following are examples of sterilization critical process parameters:

- Distribution (maximum, minimum, profile) of the physical or chemical agent throughout the entire cycle
- Time duration (minimum and maximum for any phases of the cycle)

Post-sterilization: If all presterilization critical quality attributes and sterilization process parameters are within their validated ranges, then all product units should be accepted as sterile. Failure to adequately control and measure critical process parameter data within the established, validated operating ranges of the sterilization process results in the disposal of the processed product. Under these circumstances, the application of a sterility test to ensure sterility is not permitted. Repeat sterilization of product from prior processing that failed to meet critical process parameters is only acceptable when supported with appropriate validations and regulatory approvals.

Subsequent to processing, successfully sterilized product should be physically segregated from nonsterilized and nonsterile product. Facility and/or equipment design may ensure physical segregation. Furthermore, an electronic or automated system should be used to reduce or remove the potential for mix-up due to human error.

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RISK ASSESSMENT

A well-designed parametric release process is superior because the sum aggregate of risks associated with elements of the program are lower compared to those of a non-parametric program reliant upon the sterility test. Therefore, a parametric release program is founded upon a thorough assessment, understanding, and management of risk. The multiple requisite elements of the program addressing engineering, process, procedural, and human factors require several assessments of risk to account for the different failure modalities and risk factors for severity and probability. All risk assessments should include the process parameters, critical process parameters, and the means of mitigating risks that may contribute to the failure to achieve critical quality attributes. These risk assessments are required to ensure that every product unit in every sterilization load within every manufactured batch achieves the required level of sterility assurance. For new products or sterilization processes, a risk assessment should be conducted during process development. For existing product or processes, the risk assessments should include historical data evaluation.

The following risk assessments are necessary to support a parametric release program:

Presterilization product bioburden: Assessing the potential risk of high bioburden or highly resistant microorganisms (e.g., spore formers), the means of enumeration and characterization, and opportunities for and modalities of product contamination. The risk assessment should additionally include other bioburden-related risks that may not be controlled by the sterilization process, such as pyrogens, and their potential to detrimentally alter the formulation chemistry of the product.

Loading patterns: Evaluating the positioning, stacking, and distribution of product units within the sterilizer load, including the potential for damage during the loading of each load and during the sterilization process. Include the risk of product units failing to achieve sterility due to the aforementioned factors.

Container and closure: The container and closure system is an important material attribute in which risk assessment should evaluate the risk of non-integrity before, during, and after sterilization. This includes secondary packaging and the potential for damage, and event-related or time-related loss of integrity which could potentially allow microbial ingress.

Sterilization cycle: Evaluating the controls, real-time measurement (including the number, type, and position of load monitors), data acquisition, data handling, automated and electronic systems of process control and monitoring.

Product segregation: Risk assessment of product after sterilization should evaluate the means of product management and reconciliation (manual or electronic) and the physical segregation (manual or automated) of sterilized product from presterilized and nonsterilized product.

REFERENCES

1. International Organization for Standardization. ISO 15882:2008: Sterilization of health care products—Chemical indicators—Guidance for selection, use and interpretation of results.

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⟨1223⟩ VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

INTRODUCTION

This chapter provides guidance on the selection, evaluation, and use of microbiological methods as alternatives to compendial methods. To properly implement alternative methods, one must consider a number of important issues before selecting the analytical technology and qualifying that method with the actual product. These issues include, but are not limited to, identification of suitable alternative methodology, development of user specifications for equipment selection, demonstration of the applicability of the method as a replacement for a standard compendial method, and qualification of the method in the laboratory.

This chapter outlines:

- User requirements
  - Instrument qualification
  - Validation of alternate technologies
  - Method suitability
- The limitations of the use of CFU as a standard signal for microbiological methods.
- Four novel options for demonstrating equivalence
  - Acceptable procedures
  - Performance equivalence
  - Results equivalence
  - Decision equivalence
- Application of the concept of non-inferiority to method validation
- Guidance on statistical methods that may be employed in method validation

A glossary of the terms used in the chapter is provided at the end of this chapter.

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Microbiological methods, other than microbial identification and strain typing methods (discussed in *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩), described in the compendia fall into two general categories:

1. Qualitative methods (not enumerative) that are used to assess the general microbial quality of compendial articles. This category includes assays that are intended to demonstrate the presence or absence of microorganisms in a compendial article.
2. Quantitative methods that yield a numerical (enumerative) result in terms of the microbial content of a compendial article.

There are inherent analytical factors that must be considered in the implementation of microbiological methods and in the comparison of a candidate alternative method to an existing compendial method. With respect to qualitative ("absence of") analysis, it is critical to consider that in microbiology, the finding of "no microorganisms present" does not mean in absolute terms that zero cells are actually present in the compendial article. A result of "no growth" in a current compendial method is properly interpreted as "no growth was detected in the test sample from the compendial article under the specified conditions".

The actual limits of detection of compendial microbiological methods have never been established quantitatively, and it is understood that many variables can affect the recovery of microorganisms. These variables include selection of growth media, incubation conditions, nutritional requirements of microorganisms that may be present, physical condition of microorganisms, and characteristics of the compendial article under test. Studies on the recovery of microorganisms from potable and environmental waters have demonstrated that traditional plate-count methods reporting cell count estimates as colony-forming units (cfu) may recover 0.1%–1% of the actual microbial cells present in a sample (1), in comparison to alternative methods that use flow cytometry and therefore yield a different signal (cell count). The presence of a greater number of cells based on an alternative method with a signal other than cfu has not correlated with more user risk or a higher likelihood of pathogens being present when there is an established safety record. These results do indicate that in some types of samples, the mean estimated cell count recorded using a growth-based compendial assay may result in a very different mean value than a cell count estimate derived from an alternative microbiological method that relies on a signal other than the cfu. Also, one must consider that in analytical microbiology the concept of false positive or false negative results are both scientifically and conceptually difficult. It would not be appropriate, for example to consider in a comparison between a standard compendial method and a candidate alternative method that a negative result in the standard method meant that positives observed in alternative method were false positives. It is a normal characteristic of a conventional growth-based method to recover some species well and yet to be unable to recover others. While it is not necessary for an alternative method and the conventional method to produce a match in terms of result, what is important is that the candidate method be capable of allowing a microbiologist to make an equivalent decision regarding product quality consistently.

It is extremely important in the application of this chapter that users take into account that microbiology is a logarithmic science. While we can distinguish between 100 and 1000 cfu (a difference of 1 $\log_{10}$), it may be not possible to discern smaller differences (less than 0.3–0.5 $\log_{10}$). The inherent variability of these methods is substantially greater than analytical chemistry methods. This inherent analytical variability must always be considered in the selection, development, and validation of alternative methods. The expectation of a degree of agreement between alternate microbiological methods and traditional growth-based methods beyond what is technically feasible could complicate the implementation of newer analytical technologies regardless of their specific mode of analysis.

Achieving the level of characterization (variability of the method) that is possible using modern chemical methods (e.g., high-performance liquid chromatography with a precision of 1%–2% relative standard deviation) is not possible in microbiology. It is reasonable to consider that the typical level of precision will typically be on the order of 15%–35% relative standard deviation, although results outside this range both on the high and low sides are certainly possible. Also, the enormous numbers and diversity of potential microorganisms as well as the inherent variability of metabolic activity levels in nature can complicate recovery. The advent of alternative microbiological methods, which in some cases may recover higher cell counts than typically observed using existing compendial methods, should not be taken to mean that new patient risks now exist that had not been heretofore recognized.

USP Perspective on Implementation of Alternative Methods or Procedures

The *U.S. Pharmacopeia* (*USP*) has long provided mechanisms for the implementation of alternative assay methods or procedures to analyze compendial articles. *General Notices, 6.30 Alternative and Harmonized Methods and Procedures* states, "Alternative methods and/or procedures may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances." This statement allows considerable user latitude in the decision to use an alternative procedure for routine product release, provided that proper technical and scientific attention is paid to the selection, qualification, and implementation of the method. If a product has proven safe in widespread use when released or controlled using current methods, the implementation of an alternative method which can be well-correlated to the existing method should be straightforward.

USP Methods and Procedures as Referee Tests

All methods and procedures described in the *USP* general chapters on microbiological tests, which are numbered below ⟨1000⟩, are intended to be referee tests for any product legally marketed in the United States. This means that in the event a dispute should occur for any reason, only the result obtained using the method or procedure published in *USP* is conclusive. Thus, alternative methods or procedures implemented and qualified by a user will not serve as a legal replacement for the official *USP* method, which will continue to serve as the referee test in the case of a dispute.

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General Considerations Regarding Quality Control Product-Release Assays

Although the methods and procedures described in the *USP* general chapters are intended to be official referee test methods, it is recognized that these tests may not function optimally as quality control tests for specific compendial articles without modification. It is expected that the official *USP* methods will require evaluations to determine their suitability for use when applied to specific products, and that such evaluations will be conducted on the basis of a user's specific product knowledge. Procedures for demonstration of this method suitability (verification; inhibition/enhancement) are provided in the relevant compendial test chapters. For example, some compendial articles may have inherent antimicrobial properties that could, if not modified (or neutralized), adversely affect the suitability of a given compendial method or procedure. When these methods or procedures are included in regulatory filings and the product is approved, they will be used as product-release and shelf-life tests.

Harmonized Chapters

Some *USP* microbiological methods or procedures contain a statement that they have been harmonized with corresponding methods or procedures found in the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. In chapters containing this statement, the text describing the method or procedure is considered interchangeable (for additional information, see *Pharmacopeial Harmonization* ⟨1196⟩). If a compendial method or procedure is conducted using harmonized text as written in the *USP*, *European Pharmacopoeia*, or *Japanese Pharmacopoeia*, it is considered legally interchangeable. However, the implementation of an alternative method as a quality control test to replace a method described in the *USP* does not mean this method or procedure would be interchangeable from the perspective of all relevant jurisdictions unless it meets the criteria for alternative methods as specified by the other compendia.

Submission of Alternative Methods or Procedures to *USP*

In the *USP*, it is stipulated by *General Notices, 6.30 Alternative and Harmonized Methods and Procedures* that alternate methods should be submitted to the *USP* for evaluation, if an organization wishes to have the alternative method to be considered for inclusion as a compendial method. This opportunity to advance microbiological testing is often overlooked by USP stakeholders. Submission of alternative methods or procedures allows USP to consider any such method as an addition to or replacement for an existing, standard method or procedure. A submission of an alternative method must include complete analytical and equipment details, as well as detailed analytical data from relevant qualification trials.

For any method or procedure to be considered as a replacement or additional referee method, it must not be a patented method or procedure with reagents or instrumentation available from only a single source. Also, any candidate replacement or additional method must have broad applicability, suitable for routine use and must be compatible with a broad spectrum of relevant compendial articles.

Additional *USP* Chapters Germane to the Implementation of Alternative Microbiological Methods

Several *USP* general information chapters provide usual information regarding the implementation of alternative methods and procedures. *Validation of Compendial Procedures* ⟨1225⟩ provides detailed information on submissions of alternative methods to the compendia as well as general guidance on validation and the evaluation of analytical performance characteristics. Useful information regarding the validation of methods used for the recovery of viable microorganisms from compendial articles (neutralization of antimicrobial properties of articles) appears in *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩. Information on the qualification of microbial identification methods may be found in ⟨1113⟩. Finally, information on the qualification of analytical equipment can be found in *Analytical Instrument Qualification* ⟨1058⟩.

Although *Analysis of Biological Assays* ⟨1034⟩ is primarily concerned with the determination of product potency by utilizing bioassays, it also discusses fundamental principles regarding variance, error, and biometrics that may be informative to those who are developing and validating alternative microbiological methods or procedures.

Other Information and Regulation Regarding the Use of Alternative Methods

In the U.S. Food and Drug Administration (FDA) Current Good Manufacturing Practices regulations, 21 CFR Part 211.194 describes requirements for test methods utilized to assess the compliance of pharmaceutical articles with approved specifications. The regulations state that test methods must have suitable capability regarding accuracy and reliability. This subsection of the regulations also recognizes the legal basis of *USP* and the *National Formulary* (*NF*) standards and makes it clear that it is the responsibility of the user to validate methods or procedures that differ from those standardized in the compendia.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) document Validation of Analytical Procedures: Text and Methodology [Q2(R1)] also may be a useful reference in the development and validation of alternative methods or procedures. This ICH document provides information useful in the submission of analytical procedures in association with product registration applications submitted within the United States as well as Japan and Europe.

USER REQUIREMENTS

Determining the precise technical requirements necessary for an alternative microbiological method is essential before one can select the appropriate technology and equipment that meet the relevant assay requirements. Therefore, it is suggested that organizations wishing to develop and validate a candidate alternative method produce a user requirement specification (URS)

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document. This document should include all critical functions of the technology, critical user interface requirements, space requirements, environmental requirements, operational requirements, and all other important characteristics of an alternative method for the intended use. These requirements will be specific to the company or organization, as well as to the alternative method's intended use, and therefore the requirements should be generated by the user.

In generating this URS document, three separate components of the alternative microbiological method validation must be considered:

1. *Instrument qualification.* Most alternative microbiological methods will depend on specific equipment. This analytical equipment is subject to industry standard instrument qualification requirements (see ⟨1058⟩ for further information).
2. *Validation of alternate technologies.* The basic rationale for using an alternative methodology is to improve on some aspect of the existing technology of the current compendial method without sacrificing essential characteristics of that technology (e.g., plate count and membrane filtration). The current technology for compendial microbiology methods consists of detection of the growth of viable microorganisms on (or in) a nutrient medium. The alternative technology must be at least equivalent to the current technology in terms of performance for the intended use. Much of the technical support for equivalence may come from the peer-reviewed scientific literature or from a prior regulatory submission (e.g., a vendor submitted the Drug Master File to the FDA, or prior submission from a company on this technology), but this must be confirmed, as appropriate for the intended use.
3. *Method suitability.* This consideration must address both the technology's suitability to the specific test and the lack of product inhibition and enhancement on the test results.
   A. Suitability of the technology to the specific test. Many compendial microbiological tests have mandated test requirements. An example of this would be sample plans consisting of the quantity of material to be tested (e.g., 10 g or 20 units of a specific volume). Because the test results are frequently used to determine compliance with finished product specifications, and the specifications are dependent on sample volume or quantity, the alternative technology must be able to satisfy sample volume requirements as required in the general test method. The use of a lesser volume or sample size is not recommended and would need to be fully justified by the user on a case-by-case basis. The alternative technology is considered suitable if it can meet all critical parameters of the compendial test.
   B. Inhibition and enhancement. Specific products may interfere or enhance the signal of different measurement technologies to the specific signal of interest (see ⟨1227⟩). This component of alternative microbiological method validation (i.e., suitability) must be demonstrated for each product tested.

Components of Data Quality

General information chapter ⟨1058⟩ describes four different components of data quality. The most fundamental component is qualification of the instrument; that is, a demonstration that the instrument is functioning as designed. Next in significance is the method validation; a demonstration that the technology is functioning as expected. For instance, this might be a demonstration that an alternative microbiological method is at least as suitable for its role in the test method as was the traditional plate count or recovery in nutrient broth. Next in importance is the inclusion of relevant controls in the test to demonstrate the ongoing suitability of the test system. The final component of data quality is the use of quality control samples, a practice not commonly used in microbiology because analysts strive to exclude live cultures from a product testing area. These different components of data quality are an important consideration in validating an alternative microbiological test as they help frame the URS.

Classical Microbiological Methods

The cfu has been in use for about 125 years and continues to be specified as the unit of microbial enumeration in all current *USP* monographs. However, it is important to understand that the cfu has always been an *estimation* of microorganisms present, rather than an actual count. The conceptualization of cfu as a signal requires a fundamental grasp of the process of plating bacteria, yeast, or mold on solid media, as well as knowledge of what is required to produce a single colony.

The plate count method provides an estimate of the number of microorganisms present based on the growth of discrete countable colonies on an individual plate; thus, the plate count is not a true cell count. Although it is theoretically possible for a single viable cell to give rise to a cfu, a single cell growing into a colony on a plate is unlikely to happen in nature. "Viability of a cell" is defined as the ability to multiply by binary fission such that a colony appears. For a colony to appear, viable cells must find specific conditions of nutrient growth medium, incubation, and time. Individual cells, however, are a rarity in nature, and it is far more likely that any colony growing on solid media arose from a clump, chain, or mass of cells deposited together. The cfu signal then is prone to underestimate the actual number of cells present in a sample. The extent of underestimation will vary, depending on the nature of the microorganism and the way in which the sample was prepared.

The cfu signal is also completely dependent upon recovery of microorganisms from environmental conditions, which produce stress on the organism's ability to survive. It is important to note that all organisms that are outside their preferred environmental niche will be to one degree or another stressed. Furthermore, outside laboratory culture microorganisms are unlikely to be in the exponential growth phase most optimal for transfer onto microbiological media for recovery and growth. In a very real sense most microorganisms present in compendial articles, in dry, nutrient-free environments, at elevated temperatures, high ionic strength, pH extremes, or in the presence of antimicrobial chemicals are severely stressed and may prove difficult or impossible to recover. Clearly these stress factors play a role in the plate count anomaly mentioned above. If the growth, nutritional, or incubation conditions presented to microorganisms are not sufficient to result in recovery and the growth of colonies, the signal may be 0 cfu, or no growth, even when viable cells are present. These stress factors may not be important considerations with nongrowth-based alternate methods and such methods may produce signals from cells that will not grow on media. Precision can be compromised further when organisms are present in large clumps, often associated with organic material, and are broken into smaller units during preparation. In this case, depending on the processing or handling of the

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sample, a clump could appear as a single colony or multiple colonies. Furthermore, the number of cfus on a plate must be in a countable range, for example bacteria, 25–250, for reasonably reliable enumeration.

Thus, the methods of growth-based microbiology represent a logarithmic science with a signal of enumeration (cfu) that is truly an estimate rather than a precise cell count. Understanding the strengths and weaknesses of the cfu as a signal is vital in the validation of an alternative method that uses an alternative signal. Therefore the cfu cannot be considered the only unit of microbiological enumeration.

Signals from Alternate Microbiological Methods

Rapid or modern microbiological methods typically produce signals in units other than cfu for microbial estimation and enumeration. These signals are often processed via instruments rather than visually. Extensive studies have been conducted on the capabilities of the various methods that can be applied to microbial assessment of compendial articles, and in most cases the prospective user will know the characteristics of the method and the signal it produces before selecting that method as an alternative. Guidance on method selection is provided in the section on *Validation of Alternate Technologies* and in peer-reviewed scientific publications.

Most of the rapid microbiological methods are, to some extent, direct cell count methods. They, therefore, may provide a higher cell count estimate than the cfu method for a given sample, depending on how the method is used and which compendial article is under evaluation.

Some alternate or rapid methods detect and estimate cell counts on the basis of metabolic activity, which gives rise to a signal that can be measured instrumentally. Examples of these types of signals include adenosine triphosphate (ATP) content (bioluminescence), laser-induced fluorescence, enzymatic activity, and physiochemical changes to the composition of a nutritional broth or the headspace above the broth.

Alternative methods may also be based on vital staining, in which cells are stained or exhibit autofluorescence (based on cell components) and then are directly counted, either microscopically or instrumentally. To increase the probability that only living cells will be counted, multiple stains may be used, which can (1) increase sensitivity based on cell membrane function, (2) enhance reaction with nucleic acids, or (3) improve detection of metabolic activity.

There are also nucleic acid based methods that can be used, as well as a range of other physicochemical methods of analysis that have been utilized in pharmaceutical, biopharmaceutical, clinical, and food microbiology. These methods may target, amplify, detect, or quantify a nucleic acid sequence, and it is important to understand the type of signal that results from the analytical method. In addition, one should understand the physiological characteristic of the microorganism that gives rise to the signal, which then makes it possible to enumerate the cells.

SUCCESS CRITERIA

Alternative methods for obtaining a cell count may provide higher or lower cell counts than those provided by traditional compendial methods during the enumerative analysis of compendial articles. However, whether the cell counts are higher or lower with the alternative method, it is generally possible to detect adverse trends in comparison with the estimates obtained using a compendial method.

Observations of cell counts that differ from cfu results are not a concern if the different methods and their different signals of cell presence are equivalent to or are non-inferior to referee methods in terms of assessing the microbiological safety of an article. Higher cell counts must not be considered as necessarily indicative of greater risk given the inherent variability of standard growth methods and the physical and chemical nature of compendial articles subject to analysis. This is especially true when enumerating microorganisms in articles that have a long history of safe and effective use. In such cases the discovery that an article contains a higher cell count then previously known does not mean that its safety has deteriorated.

With qualitative methods, i.e., presence or absence, comparisons of false negative and positive results obtained in controlled studies with the compendial and alternative microbiological method may be a measure of equivalence. There are commercially available enhancements to growth-based methods that allow colonies on solid media to be read more quickly, with substantially less incubation time, than is possible using only the unaided eye. These methods still require the growth of organisms on or in media. Therefore, many of these methods are not in the strictest sense different from the existing compendial methods, but are instead merely enhancements providing a more rapid detection of colonies. In the implementation of these enhanced methods for the detection of colony growth only the detection capability of the method requires verification.

SAMPLE SIZE

Any alternative microbiological test method (within its intended purpose) may use any sample size and number of tests that is sufficient to produce an equivalent decision (or better) regarding microbiological quality as compared to the reference method. It may be simpler, for many if not most alternative methods, to comply with the sampling instructions that are provided in the official compendial method. In case the sampling approach defined in the official compendial method is utilized no justification of the sample size is required.

Statistics and Alternative Methods

Attempts to use statistics to compare the cfu results to signals arising from biochemical, physiological, or genetic methods of analysis may have limited value. Given the differences among these methods, they cannot be expected to yield signals that could be compared statistically in terms of mean values and variability. Thus, the enumerative values, given as cfu results in association with reference methods, typically cannot be used as acceptance criteria for the assessment of articles via candidate alternative methods. Instead, it is the users' responsibility to propose values, supported where necessary by scientific literature, that they can demonstrate are appropriate for the method that they have chosen and validated. This can be done independently of existing standards expressed in terms of cfu.

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INSTRUMENT QUALIFICATION

Instrument qualification should follow, at least in general terms, the discussion in ⟨1058⟩. The instrument qualification for equipment critical to the functioning of an alternative microbiological method involves four distinct phases:

1. Establishment of User Requirement Specifications for critical method attributes which should be formalized in a URS document.
2. Installation qualification—Was the instrument installed correctly?
3. Operational qualification—Does the instrument meet the manufacturer's specifications for correct operation?
4. Performance qualification—Does the instrument meet the URS for performance?

VALIDATION OF ALTERNATE TECHNOLOGIES

User Requirements Specification

Preparation of the URS document should involve input from all stakeholders for the microbiological test method. These stakeholders may include representatives from the Microbiology, Quality, Regulatory Affairs, and Operations groups, as well as others. The time spent on this step should be considered an investment in reaching a clear understanding of the company's needs before equipment is purchased, which will drive the performance qualification. At minimum, this document should include the following:

- Purpose and intended use (defined need for instrument)
- Description of who will use the equipment
- Operational requirements (data format, user interfaces, and operating environment)
- Constraints (timetables, downtime, maintenance, user skill levels, product compatibility, limit of detection, accuracy, and rapidity)
- Life cycle (development, testing, delivery, validation, training, and obsolesce)
- Capability (turnaround time, test capacity and throughput, and labor requirements)
- Sustainability (consumables, calibration, validation, and preventative maintenance)

See ⟨1058⟩ for information on the qualification of analytical instrumentation. The principles outlined in ⟨1058⟩ are generally applicable to the qualification of instruments used to conduct alternative microbiological analysis. The user may need to tailor the specific recommendations in ⟨1058⟩ to their particular instrument qualification specifications.

Validation Criteria

The validation parameters generally recommended for qualitative and quantitative microbiological tests are shown in *Table 1*. Examples of qualitative tests are the sterility test and the test for absence of specified microorganisms. A quantitative test would be microbial enumeration. Note that qualitative testing is binary, and for this reason there is generally no need to define equivalency of units of measure, only equivalency of outcome.

Table 1. Validation Parameters by Type of Microbiological Test

Validation Parameter	Qualitative Tests	Quantitative Tests
Accuracy	No	Yes
Precision	No	Yes
Specificity	Yes	Yes
Limit of detection	Yes	Yes
Limit of quantification	No	Yes
Linearity	No	Yes
Operational (dynamic) range	No	Yes
Robustness	Yes	Yes
Repeatability	Yes	Yes
Ruggedness	Yes	Yes
Equivalency	Yes	Yes

SPECIFICITY

Definition: The specificity of an alternate qualitative microbiological method is defined as its ability to detect a range of challenge microorganisms specific to the technology. "Range of microorganisms" may be defined as a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are representative in terms of morphological and physiological attributes appropriate for the method and the product.

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Demonstration: Specificity is demonstrated by comparable recovery of the challenge panel of microorganisms in both the compendial and alternate methods. The microbial challenge is above the limit of detection or quantification but at a level that provides a measure of efficacy of the methods.

Growth based—Add low numbers (around 100 cfu) of each microorganism on the panel and perform both the compendial and alternative methods to demonstrate recovery of the microorganism.

Nongrowth based—Use suitable negative and positive controls to demonstrate that extraneous matter that may be in the system (e.g., extracellular ATP, DNA, or inhibition and enhancement factors) does not interfere with the detection of the defined range of microorganisms.

All challenge microorganisms should be recovered and identified in growth-based methods. For nongrowth-based methods microorganisms should be recovered and identified where possible.

LIMIT OF DETECTION

Definition: The limit of detection (LOD) of an alternate microbiological method is defined as lowest number of microorganisms in a defined volume of sample that can be detected, but not necessarily quantified, under the stated experimental conditions. This should be conducted with the quality control organisms cited in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Mycoplasma Tests* ⟨63⟩, and *Sterility Tests* ⟨71⟩ as appropriate to the alternative method.

Demonstration

Method 1

- Inoculate a suitable diluent solution with a serial dilution range of each challenge microorganism, appropriate for the intended use of the method and the technology. In most cases, the compendial media growth promotion test panel may be sufficient.
- The level of inoculation should be adjusted to a target of 50% of the dilution samples that show growth in the compendial test.
- Perform both the compendial and alternate tests.
- Tests should be repeated a sufficient number of times (statistically significant alpha risk: 0.05; beta risk: 0.20) for both the compendial and alternate tests.
- *Statistics:* Use the chi-square test or another appropriate approach to demonstrate equivalent recovery of the microorganism challenges.
- Alternately, use *Method 2*.

Method 2 (MPN Method)

- Create a dilution series of the challenge organisms in a suitable diluent solution to include at least the range of 10^{1} cfu to 10^{-2} cfu (for a 10-fold series) or 5 cfu to 10^{-1} cfu/inocula volume (for a 2-fold dilution series).
- Perform both the compendial and alternate tests with at least 5 simultaneous replicates of each dilution from the chosen series.
- Determine the most probable number (MPN) from three dilutions in series that provide both positive and negative growth (or signal).
- Statistics: Use the chi-square test or another appropriate approach to demonstrate equivalent recovery of the microorganism challenges.

ROBUSTNESS

Definition: A capacity of the method to remain unaffected by small but deliberate variations in method parameters, e.g., reagent volume, incubation time, or ambient temperature providing an indication of its reliability during normal usage. A measure of robustness is not a comparison between the compendial and alternate methods; rather, it is a necessary component of validation of the alternate method so that the user understands the limits of the operating parameters of the method. The user may rely on data supplied by test method manufacturer.

RUGGEDNESS

Definition: The degree of precision of test results obtained by the analysis of the same samples under a variety of typical test conditions such as different analysts (for example, three), instruments, and reagent lots (the method for demonstration may follow instrument or materials supplier recommendations, or it could be based solely on data supplied by test method manufacturer).

For the definition of other validation parameters see *Glossary*.

METHOD SUITABILITY

For each new product to be tested using the validated alternate microbiological method, perform the suitability test as described in general test methods (see ⟨51⟩, ⟨61⟩, ⟨62⟩, ⟨63⟩, and ⟨71⟩), using the number of unit and quantities prescribed and the sample preparation appropriate for the product and the required test sensitivity to determine the absence of a product effect that would obscure the signal of the method.

Method suitability may be demonstrated using three independent tests. Only the accuracy and precision validation parameters are required for quantitative methods. For qualitative methods, recovery of challenge organisms as indicated in ⟨62⟩, ⟨71⟩, and ⟨1227⟩ is sufficient.

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After an alternative method has been shown to be equivalent to the compendial test with one product, it is not necessary to repeat the equivalency parameters for every new product; it is merely necessary to verify the method suitability for each additional product. For example, when employing a nucleic acid based method, with each new product, one must demonstrate that residual product does not interfere with the concentration, extraction, purification, and recovery of the target nucleic acid, or the polymerase chain reaction (PCR) amplification and chemical probe detection of the target ribosomal ribonucleic acid (rRNA) gene sequence.

EQUIVALENCY

All microbiological tests are performed to enable informed decision making regarding the microbiological quality of a product, raw material, component, or process step. In this respect, the intended purpose of microbiological tests may be to either evaluate for the presence or absence of microorganisms (as in the sterility test) or to estimate the number of organisms present. The technological means by which microbiological test methods assess microbiological quality and enable a product-quality decision may differ from the growth-based means typical of reference methods. The units of measurement (signal) of a microbiological quality assessment performed using alternative microbiological test methods will generally not be a cfu, but rather a different approach to obtaining a cell count estimate. Therefore, the validation of alternative microbial methods should involve two components: (1) equivalence demonstration and (2) analytical method and equipment qualification.

Equivalence Demonstration

General Notices, 6.30 Alternative and Harmonized Methods and Procedures states that alternate methods may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction. It further stipulates that alternative methods can be implemented in other special circumstances. Such alternate methods shall be validated as described in ⟨1225⟩ and must be shown to produce equivalent or better results than the referee method for any given quality attribute. When comparing two test procedures to show equivalent or better performance, statistical evidence is assembled to show equivalence or, in statistical terms, non-inferiority. For example, with microbial enumeration, equivalency may be shown if there is no statistically significant difference between the two means generated when enumerating with the compendial and alternative methods. However, this may not be possible when the two methods yield different signals. Examples of this situation are when the microbial enumeration method uses vital staining of microbial cells or measurement of genomic material in place of cfus.

Similarly, the FDA *Guidance for Industry* document *Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls Documentation* states that a validated alternative analytical procedure should be submitted only if it is shown to have performance equal to or better than the regulatory analytical procedure. Also, section 2.7 of the ICH document *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Q6A)* states that alternative procedures are those that may be used to measure an attribute when such a procedure controls the quality of a drug substance or drug product to an extent that is comparable to or superior to the official method. However, other options to demonstrate equivalence are available and are discussed in *Demonstration of Equivalency*.

Demonstration of Equivalency

Four options are available to establish the equivalence of a candidate alternative analytical method: (1) acceptable procedures (i.e., merely meeting a minimum performance or acceptance requirement without a need to demonstrate equivalence to the compendial method); (2) performance equivalence to the compendial method; (3) results equivalence to the compendial method; and (4) decision equivalence to the compendial method (*1*). A comparison of these four equivalence options is given in *Table 2*. The multiple equivalence options reflect the diversity in the technology and applications of the alternative test methodologies and may be viewed as a paradigm shift in *Equivalence Demonstration*.

Table 2. Equivalence Option Matrix

Option	Demonstration	Comparison to Official Compendial Method	Based on Numerical Results or Conclusion	Number of Characteristics
1. Acceptable procedures	Acceptable	No	Results	Multiple
2. Performance equivalence	Equivalent	Yes	Results	Multiple
3. Results equivalence	Equivalent	Yes	Results	Single
4. Decision equivalence	Equivalent	Yes	Conclusions	Single

OPTION 1: ACCEPTABLE PROCEDURE

This is not strictly an equivalence option that requires direct comparison between the candidate alternative method and an official compendial method. With this option, a reference material with known properties may be used, such as a standard inoculum of a specific microorganism, a quantity of highly purified bacterial genome, an ATP level or another appropriate signal. In some cases, it could be required that the alternative method measure the signal in the presence of the test sample, with validation criteria that are consistent with the capability of the technology, as described in the scientific literature.

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OPTION 2: PERFORMANCE EQUIVALENCE

Performance equivalence requires the demonstration of equivalent or better results with respect to validation criteria—such as accuracy, precision, specificity, limit of detection, limit of qualification, robustness, and ruggedness—that may be appropriate for the intended use of the alternative qualitative or quantitative method. It is possible that the alternative method may not conform to some of the validation parameters listed compared with the official method and still be acceptable because of the advantages of the alternative method. This may be the case if the alternative method has any of the advantages stated in the *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*. Other special circumstances would include improvements in time to obtain a result or the cost of running the test. If a candidate alternative method is suitable for assessing the quality of the material tested, it may be still acceptable, even if it differs from the official method in one or more validation parameters. The final analytical qualification criteria should reflect only the criteria that the microbiologist deems necessary to achieve performance equivalence.

OPTION 3: RESULTS EQUIVALENCE

When results equivalence is required, the hypothesis to be tested is that the alternative and compendial test methods give equivalent numerical results. This contrasts with the evaluation of the validation parameters, as is done in performance equivalence. Because the same sample cannot be tested in microbiology, typically a tolerance interval is established when comparing the two methods, with the alternative method determined to be numerically superior or non-inferior. Reports on the use of alternative non-growth-based methods have shown that they may produce significantly higher cell count estimates than a growth method that reports outcomes in cfu. In this case, the analyst could use a calibration curve showing a correlation between the two methods in the product specification range.

OPTION 4: DECISION EQUIVALENCE

A decision equivalence is similar to results equivalence but differs in that a numerical result is not generated; instead a pass/fail result is obtained. With this approach, the frequency of positive and negative results generated should be no worse than with the compendial method. This non-inferiority requirement is based on the long history of product quality tested by and released with the referee compendial test. For the purposes of qualification, laboratory studies involving spiking low levels of microorganisms may be considered. The following sections provide suggested approaches for demonstrating that the alternative procedure is equivalent to or better than the compendial procedure. Users may use other valid methods for demonstration of equivalence with supporting scientific justification.

Equivalence Demonstration for Alternative Qualitative Microbiological Procedures

Results obtained by procedures in ⟨62⟩, ⟨63⟩, and ⟨71⟩ are indicative of the presence or absence of microorganisms in the sample tested. These tests do provide a decision (i.e., the compendial article either passes or fails the test). This type of data fits in the decision equivalence category as described in the *Stimuli* article (*2*). *Approach 1* (see below) is based on demonstrating decision equivalence. *Approach 2* (see below) is an alternative that converts the qualitative results to quantitative ones by using the most probable number (MPN) procedure. Both approaches use a non-inferiority hypothesis (*3*).

To demonstrate the acceptability of the alternate procedure relative to the current microbiological procedure, the laboratory must demonstrate that the new procedure is as good as or better than the current procedure in terms of the ability to detect the presence of microorganisms. In general, a recommended approach for comparing the alternate procedure to the compendial procedure is to use a non-inferiority test (one-sided, as in non-inferiority tests conducted in clinical trials for new drug products) (*4*) rather than two-sided equivalence [as in bioequivalence (*5*)]. Non-inferiority is an appropriate approach for two reasons. First, from a patient perspective, it is beneficial to promote an alternative procedure that is potentially more sensitive than the referee procedure. In contrast, a two-sided approach penalizes better recovery of microorganisms. It is important to note that those implementing the alternate method will need to assess the risk associated with the change in procedure, because a more sensitive procedure may generate more positive results. Second, the alternate procedure has benefits (principally, reduced time to a result) that make it preferable to the compendial procedure, even if it is not as sensitive, as long as it allows for a quality decision on the product that is non-inferior to the compendial method.

APPROACH 1: USE PRESENCE AND ABSENCE RESULTS

The non-inferiority hypothesis for this approach is that the proportion of samples that produce a signal for the new procedure (P_N) is NMT some amount ($\Delta > 0$) less than the proportion for the current, compendial procedure (P_C) (*6*):

$$\text{Result} = P_N - P_C \geq -\Delta$$

The Δ is the non-inferiority margin. Unless the laboratory requires a tighter margin, use $\Delta = 0.20$ in the experiments described below. Calculate a one-sided 90% confidence interval for $P_N - P_C$ (*7*). Non-inferiority is concluded if the lower confidence limit exceeds –0.20. If the experiment is able to conclude in favor of the non-inferiority hypothesis, then it can be stated, with 95% confidence, that $P_N \geq P_C - 0.20$ at the bioburden level studied.

This evaluation should be conducted using types of microorganisms selected by the laboratory as representative of the general types of microorganisms encountered. The choices can follow ⟨71⟩ or appropriate suitability test organisms, organisms recovered from product testing and/or microorganisms representative of those that may convey risk to patients given a product's route of administration.

The laboratory should conduct an evaluation to determine whether the alternate procedure can be shown to be non-inferior to the microbiological procedure in terms of sensitivity as measured by the proportion of samples returning a positive result for

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microbial recovery and growth. For each organism in a qualitative test, conduct three evaluations. The first uses samples prepared by serial dilution to be at or around 10^0, i.e., 1 cfu, where no growth is likely to be observed (hence no signal will be detected by the growth-based microbiological procedure) to characterize the sensitivity of the new procedure at this level. The second uses samples at or around 10^2 (100–200 cfu), where the microbiological procedure would be expected to detect growth at a relatively high percentage of about 75% or greater, to determine the acceptability of the new procedure. The third is a comparison of the two procedures at a burden where 50%–75% of samples would be expected to grow colonies [often a serial dilution to around 10^1 (10–50 cfu)] to test the non-inferiority hypothesis as described above. In the non-inferiority experiment for qualitative microbiology tests, a minimum of 75 samples should be tested on each procedure. Using 75 samples provides approximately 80% power. Should the laboratory find that a higher statistical power is necessary given the requirements of the analysis for a given product, increasing the number of samples analyzed to 100 will result in a power of approximately 90%. These procedures are appropriate for the purposes of concluding non-inferiority if the two procedures are actually equally sensitive using $\Delta = 0.20$. If the laboratory concludes their new procedure is less sensitive than the compendial procedure, a larger number of samples will be required to maintain these power levels.

Independent samples: Suppose that N_A samples have been tested with the candidate alternative procedure, of which X_A samples are positive, and that N_C samples (not the same as those tested with the candidate) have been tested with the compendial procedure and that X_C samples are positive. Calculate the following:

$$\hat{p}_A = X_A / N_A \quad \hat{p}_C = X_C / N_C$$
$$\theta = N_C / N_A$$
$$a = 1 + \theta$$
$$b = -\left[R(1 + \theta\hat{p}_C) + \theta + \hat{p}_A\right]$$
$$c = R(\hat{p}_A + \theta\hat{p}_C)$$
$$\tilde{p}_A = [-b - (b^2 - 4ac)^{1/2}] / (2a)$$
$$\tilde{p}_C = \tilde{p}_A / R$$
$$V = \frac{\tilde{p}_A(1 - \tilde{p}_A)}{N_A} + R^2 \frac{\tilde{p}_C(1 - \tilde{p}_C)}{N_C}$$
$$Z = \left(\hat{p}_A - R\hat{p}_C\right) \Big/ \sqrt{V}$$

where R is the ratio of variances at which to determine power.

Conclude non-inferiority if $Z > z_\alpha$ where z_α is the upper α percentage point of a standard normal distribution.

Paired samples: Suppose that N samples have been tested by both the candidate alternative and compendial procedures. The results can be displayed in a 2 × 2 table (see *Table 3*).

Table 3. Results for Paired Sample

Alternative Procedure	Compendial Procedure		Row Totals
	Positive	Negative	
Positive	X_{11}	X_{10}	X_A
Negative	X_{01}	X_{00}	$N - X_A$
Column totals	X_C	$N - X_C$	N

Compute the following:

$$L = [X_{10} - RX_{01} + (1 - R)X_{11}] / N$$
$$V = X_A(X_{10} + X_{01}) / X_C^3$$
$$Z = L / \sqrt{V}$$

Conclude non-inferiority if $Z > z_\alpha$ where z_α is the upper α percentage point of a standard normal distribution.

APPROACH 2: COMPARE MPN RESULTS

For the compendial reference and the alternative procedures, conduct an MPN comparative study using standard procedures for MPN for each of the N samples. Ideally, the same samples are used for the two procedures, but this is not a necessity.

For *Approach 2*, the non-inferiority hypothesis is

$$\mu_A - \mu_C \geq \log(R) \text{ or } \textit{anti}\log(\mu_A - \mu_C) \geq R$$

where μ_A and μ_C are the means in the log scale for the alternative and compendial procedures, respectively.

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Independent samples: Determine MPN for N_A samples by the alternative procedure, convert all values to logs, and determine the sample mean of the log values ($\bar{x}_A$) and sample variance of the log values (S^2_A). Similarly, determine $\bar{x}_C$ and S^2_C from the logs of N_C samples tested with the compendial procedure. Determine the following:

$$L_{low} = \bar{X}_A - \bar{X}_C - t_{\alpha,df}\sqrt{\frac{S_A^2}{N_A} + \frac{S_C^2}{N_C}}$$

where $t_{\alpha, df}$ is the upper α percentage point of the *t* distribution with *df* degrees of freedom and

$$df = \frac{(S_A^2/N_A + S_C^2/N_C)^2}{\frac{(S_A^2/N_A)^2}{N_A - 1} + \frac{(S_C^2/N_C)^2}{N_C - 1}}$$

If using software that only allows for integer degrees of freedom (e.g., Excel), use linear interpolation to obtain the *t* value. Conclude non-inferiority if *anti*log(L_{low}) ≥ *R*.

Paired data: Determine MPN for *N* samples by the alternative procedure and for the same *N* samples by the compendial procedure, convert all 2*N* values to logs, and determine the sample mean ($\bar{x}$) and variance (S^2) of the differences of log alternative value minus log compendial value. Determine the following:

$$L_{low} = \bar{x} - t_{\alpha, N-1}\, S/\sqrt{N}$$

where $t_{\alpha, N-1}$ is the upper α percentage point of the *t* distribution with $N - 1$ degrees of freedom. Conclude non-inferiority if *anti*log(L_{low}) ≥ *R*.

Equivalence Demonstration for Alternative Quantitative Microbiological Procedures

A key characteristic of some alternative quantitative procedures is that their signal may differ significantly from the cfu of the compendial microbiological procedure. As a consequence, equivalence as it is typically understood cannot be shown; that is, the numerical results are expected to differ in magnitude and units. Instead, this chapter suggest two criteria for the verification of candidate alternative quantitative procedures:

1. Results from the candidate procedure have at least acceptable precision (repeatability).
2. The results from the candidate procedure are highly correlated with those from the compendial procedure. A high correlation is taken to indicate that quantitative acceptance criteria expressed in cfu can be calibrated to criteria in the units of the alternative procedure.

PRECISION

Prepare a minimum of six samples at a minimum of two bioburden levels near specification limits relevant to the laboratory. Run the candidate alternative procedure for the prepared samples. [NOTE—This is to correspond to repeatability conditions; see ⟨1225⟩.] At each level, determine the sample variance (S^2) of the logarithms ($\log_{10}$) of sample results. Calculate the following:

$$UL = 100 * \left[anti\log\left(\sqrt{\frac{(n-1)S^2}{\chi^2_{.05,\, n-1}}}\right) - 1 \right]$$

Where *n* is the number of samples ($n \geq 6$) and $\chi^2_{.05,\, N-1}$ is the lower 5% value of a chi-square distribution with $n - 1$ degrees of freedom. Precision is acceptable if *UL ≤ σ, where σ is the predetermined maximal acceptable repeatability percent geometric coefficient of variation, %GCV. (For small values, the %GCV will be approximately the %RSD.)

The greater the number of samples (*n*) the greater the likelihood (power, in statistical terms) that a procedure, the precision of which is actually acceptable, will yield data that meet this criterion and thus be declared acceptable. The laboratory may use prior data to determine a value of *n* that meets their needs.

Example: For the data for the alternative procedure in *Table 4*, calculate the following:

$n = 10$

$S^2 = 0.000241$, and

$\chi^2_{.05,\, 9} = 3.325113$, so

UL = 6.06%

This alternative procedure thus has acceptable repeatability precision as long as the prespecified success criterion σ had been at least 6.06%.

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CORRELATION (LINEARITY)

Prepare a minimum of two samples at each of four different bioburden levels covering the range from near limit of qualification (LOQ) to one log above the specification limit defined in the standard compendial assay to which the candidate alternative method is being compared. Determine the activity for all samples using both the candidate alternative and compendial procedures. Plot and determine the correlation between the log of values from the candidate alternative procedure (y) and the log of values from the compendial procedure (x). The correlation is acceptable if at least 0.95 (or R^2 at least 0.9025).

Although a linear relationship between the two sets of results is typically expected, a nonlinear relationship can be acceptable. In the case of a nonlinear relationship, use the Spearman (nonparametric) correlation instead of the Pearson correlation.

Table 4

Compendial (cfu)	Alternative (cell count)
70	970
71	965
75	950
92	990
100	1000
105	1051
116	1046
123	1039
127	985
130	1020

Figure 1 shows the plot of these data after conversion to base 10 logs. Because R^2 does not meet the stated requirement, the results from these two procedures are not sufficiently correlated.

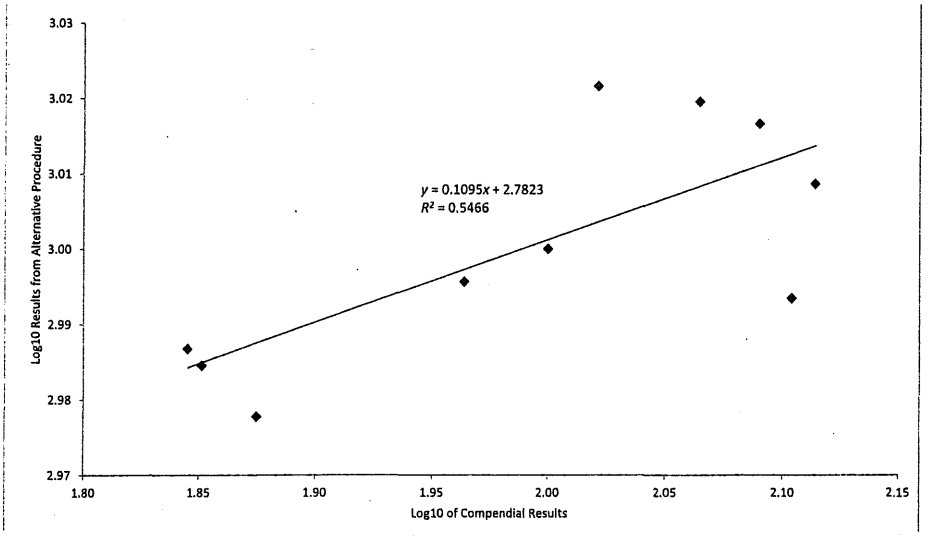

Figure 1.

Candidate alternative procedures that may be suitable for making decisions about the microbiological quality of a sample (as in *Figure 1*) may not correlate well enough with the compendial procedure to meet the above correlation requirement. In that case, the other option is to apply the decision equivalence approach as described earlier for qualitative tests. During procedure development, the laboratory should determine a specification for the alternative method to correspond to the compendial specification for the required level of microbiological quality. For example, if the required level of microbiological quality is NMT 10^2 cfu, for which the compendial maximum acceptable count is 200 cfu, the laboratory will need to determine an acceptance criterion for the candidate alternative procedure that will match that value from the perspective of making a decision regarding microbial quality. Then, the validation experiment to confirm this choice proceeds as described earlier for qualitative tests.

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GLOSSARY

Accuracy: Closeness of the test results obtained by the alternative test method to the value obtained by the compendial method, to be demonstrated across the dynamic (operational) range of the method.

Alternative microbiological method: A modern or rapid microbiological test procedure (MMM or RMM) that is different from the traditional growth-based method, such as the plate count or recovery in liquid broth. The alternative or rapid method may use different technologies, instrumentation and software to manage the testing and analyses of data and may provide quantitative (enumeration) or qualitative (detection) microbial test results.

Colony-forming unit (cfu): An estimate of the number of microorganisms obtained by traditional plate count methods. The enumeration is dependent on the ability of the microorganisms in the sample to grow on the microbiological culture media under the conditions of incubation. Because it is uncertain whether a colony was derived from the growth of one or even one thousand cells, the results are reported as cfu/mL (for a liquid) or cfu/g (for a solid) and not as cells/mL or cells/g.

Conventional microbiological method: A classical or traditional growth-based method, such as enumeration on an agar plate or detection in a liquid broth when incubated for a specified time and temperature. These methods are used in ⟨51⟩, ⟨61⟩, ⟨62⟩, ⟨63⟩, and ⟨71⟩.

Equivalence: When the test results from two procedures are sufficiently close for the intended use of the procedures. Demonstration of equivalence requires a prespecified measure of how similar the test results need to be.

False negative: A test result that is incorrectly determined as negative (e.g., the absence of a viable microbial detection result when viable microorganisms are present). A type II error, also known as an error of the second kind, occurs when the null hypothesis is false but erroneously fails to be rejected. It is failing to assert what is, in fact, present—a miss. A type II error may be compared with a so-called false negative in a test (and seen as a "miss") that is checking for a single condition with a definitive result of true or false. The rate of the type II error is denoted by the Greek letter β and is related to the power of a test (which equals 1 − β).

False positive: A test result that is incorrectly determined as positive (e.g., a viable microbial detection result when viable microorganisms are not present). In statistical test theory, the idea of a statistical error is an integral part of hypothesis testing. These are described as type I and type II errors. A type I error, also known as an error of the first kind, occurs when the null hypothesis (H0) is true but is rejected. It is asserting something exists that is, in fact, absent (i.e., a false hit). A type I error may be compared with a so-called false positive (a result that indicates that a given condition is present when it actually is not present) in tests where a single condition is tested. The rate of the type I error is called the "size" of the test and denoted by the Greek letter α. It usually equals the significance level of a test. In the case of a simple null hypothesis, α is the probability of a type I error.

Independent samples: Samples selected from the same population or different populations that have no effect on one another. That is, no correlation exists between the samples.

Limit of detection (LOD): The lowest concentration of microorganisms in a test sample that can be detected, but not necessarily quantified, under defined experimental conditions.

Limit of quantification (LOQ): The lowest number of microorganisms in a test sample that can be enumerated with acceptable accuracy and precision under defined experimental conditions.

Linearity: The ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range.

Method suitability: Demonstration of lack of enhancement or inhibition by the product on the signal generated by the method.

Non-inferiority: Demonstration that the alternate method is not worse than the compendial method by more than a small prespecified amount. This amount is known as the non-inferiority margin or δ. Non-inferiority is different from equivalence in that in an equivalence trial, the desired conclusion is that two microbiological methods are not unacceptably different from each other. In a non-inferiority test the objective is to demonstrate that a new product is not unacceptably worse than an older one.

Paired samples: A sample of matched pairs of similar units.

Range-Dynamic or Operational: The interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with specified accuracy, precision, and linearity.

Repeatability precision: The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of the same suspension of microorganisms and uses different suspensions across the range of the test. Also known as "repeatability".

Robustness: A method's capacity to remain unaffected by small but deliberate variations in method parameters, such as, reagent volume, time or temperature of incubation providing an indication of its reliability during normal usage.

Ruggedness: Intermediate (within laboratory) precision associated with changes in operating conditions. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay (e.g., different days, different analysts, different equipment).

Specificity: The ability to detect a range of microorganisms, which demonstrate that the method is fit for its intended use. These microorganisms may include a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are specified in the relevant compendial tests, that are appropriate for the method and the product.

User Responsibility: The responsibility for the installation and operational, and performance qualification may be a joint responsibility of the instrument manufacturer and the user of the alternative microbiological method. The method validation may be conducted by the instrument manufacturer, when justified. The method suitability testing conducted for each specific product must be solely the user's responsibility.

Validation: The process of demonstrating and documenting that the performance characteristics of a procedure and its underlying method meet the requirements for the intended application and that the procedure is thereby suitable for its intended use. Formal validations are conducted prospectively according to a written plan that includes justifiable acceptance criteria on validation procedures.

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⟨1223.1⟩ VALIDATION OF ALTERNATIVE METHODS TO ANTIBIOTIC MICROBIAL ASSAYS

INTRODUCTION

Microbiological assay methods have traditionally been used to quantify the potency, or antimicrobial activity, of antibiotics. These microbiological procedures were historically used to certify antibiotics on a lot-by-lot basis to ensure sufficient activity. Before 1998, monographs for several approved antibiotics were published in FDA's Code of Federal Regulations, 21 CFR. These regulatory antibiotic assay procedures were later published in *USP* as the official referee methods to determine the potency of antibiotics. The details of the microbial assay procedures for individual antibiotics, including the challenge organisms and test parameters, are described in general chapter *Antibiotics—Microbial Assays* ⟨81⟩. Microbial assays provide a direct measure of the effectiveness of the antibiotic against a reference microorganism. Although these microbiological methods have continued to serve as the official compendial referee methods since their publication in *USP*, many manufacturers have replaced these microbiological analyses with high-performance liquid chromatography (HPLC) methods.

Although the microbiological methods provide direct proof of antimicrobial effectiveness and can integrate all moieties that contribute to antimicrobial effects in a formulation, these methods are less precise, more complex to perform, and slower to complete than alternative methods such as HPLC. Microbial assays also have limited selectivity and are not appropriate for evaluating organic impurities. The specific skill sets required to perform the microbiological antibiotic assays, their unique equipment requirements, and their comparative complexity deter many stakeholders from using these methods.

There are numerous reasons for replacing the microbiological antibiotic assays with chemical assays that use purity or content as surrogates for the measurement of biological activity. The advantages of chemical-based analytical methods have been described previously for simple, single-component antibiotics, as well as complex, multi-component antibiotics (*1*). Physicochemical procedures, such as HPLC, allow for simpler preparation and rapid data acquisition with improved precision, accuracy, selectivity, and specificity. HPLC methods can be used effectively for both potency assignment and organic impurity testing. Additionally, because modern instruments and the expertise to use such equipment are widely available, the conversion to alternative methods may be economically advantageous.

This general chapter provides points to consider for manufacturers who want to use physicochemical alternatives instead of the microbial assay methods described in ⟨81⟩. Given the widespread use of HPLC as an alternative to microbial assay methods, this chapter focuses on HPLC methods. However, the principles set forth in this chapter are applicable to any alternative physicochemical procedure.

GENERAL CONSIDERATIONS FOR ALTERNATIVE METHOD DEVELOPMENT

Multiple important factors need to be considered when replacing microbial methods with HPLC or other chemical techniques; this is because of the specific characteristics of antibiotics.

1. It is essential to know whether the antimicrobial activity manifested by a preparation results from a single, active ingredient or arises from multiple, often related, moieties. Where technically possible, the analyst should determine the contribution of major moieties to antibiotic effectiveness. For the purposes of this chapter, major moieties are defined as those that contribute more than 1% of the antibiotic potency. Where multiple moieties contribute to antibiotic activity, the chemical assay should be able to resolve all major moiety peaks in the formulation.
2. To the extent possible, the individual activity of process impurities and degradation products should be evaluated. This may not be necessary where evidence exists that each of the impurities and degradation products contributes less than 1% of the total antimicrobial activity of a preparation.
3. General chapter ⟨81⟩ is the referee standard in any procedure comparison. Therefore, the currently official USP procedure should be performed by the manufacturer (or under that manufacturer's direction) to establish reference assay values

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within the test range. It may be necessary to evaluate data using the guidelines in general chapter *Analysis of Biological Assays* ⟨1034⟩ when the test design is one described in that chapter. Manufacturers should establish appropriate limits for precision and accuracy of the microbial assay procedure based upon their product knowledge.

4. The candidate alternative HPLC or other chemical method should be fully validated according to general chapter *Validation of Compendial Procedures* ⟨1225⟩.
5. The value of any method comparison is dependent upon the precision and accuracy of the assay test results obtained from both methods. No statistical comparison should be undertaken if the assay data from either method does not meet predetermined acceptance criteria for method validation.
6. The guidelines in this chapter do not apply to already marketed products for which the manufacturer has already received regulatory approval to use an alternative method. In all cases, the applicability of an alternative method can only be determined through submission and review by the relevant regulatory authority.
7. Any alternative to a compendial procedure must be validated and proven to be equivalent to, or better than, the referee method (*2*). A stimuli article published in *PF 35(3)* [May–June 2009] discusses the "equivalent or better" approach to evaluating alternatives to compendial procedures (*3*). It is expected that such a comparison will use appropriate statistical analysis. Examples of the recommended types of statistical analyses are outlined in this chapter.

TECHNICAL CONSIDERATIONS

Simple and complex antibiotics are treated separately because there are significant differences in methods development and validation for these two categories of antibiotics.

- Simple antibiotics (such as tetracycline) are those for which all antimicrobial activity is contributed by a single moiety.
- Complex antibiotics (such as gentamicin) are those that have more than one active moiety.

Stability-indicating alternative procedures are recommended. Where impurities or degradation products contribute more than 1% of the antimicrobial activity of an antibiotic preparation, impurities and degradation products must be evaluated.

The microbial assay and the candidate alternative method should be performed as described in the appropriate general chapter, e.g., ⟨81⟩ for microbial assays and general chapter *Chromatography* ⟨621⟩ for HPLC procedures.

BRIDGING STUDIES

Bridging studies are used to compare the data obtained from candidate alternative procedures to the microbial assay data to determine whether the alternative procedure is an acceptable substitute.

Simple Antibiotics

1. Separate the active antibiotic moiety from impurities and degradation products. Compare the microbial activity of the main moiety, process impurities, and degradation products against the USP Reference Standard for the antibiotic. Process impurities and degradation products present at levels below 1% of antimicrobial activity may be disregarded. The candidate alternative method may resolve impurities that do not have antimicrobial activity. These will likely not factor into the bridging study.
2. Continue as described in this section only if the product has a single moiety conveying the antimicrobial activity.
3. Validate the alternative procedure using ⟨1225⟩. The alternative procedure must be specific, selective, and stability indicating.
4. Test a minimum of three separate lots of the drug substance and the relevant USP Reference Standard using the microbial assay procedure as well as the candidate alternative method.
5. Test a minimum of six replicate samples per lot using both the microbial assay and candidate alternative method. It may be necessary to increase the number of replicates based on the maximum allowed percentage difference (see *Data Evaluation*) and the standard deviation of the method (*4*). If possible, prepare stock standard and sample solutions and subdivide them for use with the microbial assay and candidate alternative assay procedures. This provides paired data that can be analyzed (see *Appendix 2*).
6. Apply appropriate outlier (see general chapters *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and ⟨81⟩) and comparison tests (see *Data Evaluation*) to determine if the candidate alternative method and the microbial assay procedures yield equivalent results.
7. If there are statistical outliers with either procedure, perform the comparison by excluding the outlier values. If the bridging study criteria are met, repeat the bridging study with controls to prevent outliers. Justification for rejection of outliers must be provided in the bridging study report. The consistent appearance of outliers in the candidate alternative assay results may indicate that the procedure has inadequate controls. If this occurs, the bridging study is void and a different alternative procedure should be developed and validated.
8. If the bridging study fails, it may be necessary to include potency contributions from impurities that are below 1%.

Complex Antibiotics

1. Separate and purify each antimicrobial moiety of a complex antibiotic preparation, process impurities, and degradation products. Compare the antimicrobial activity of the main moiety, process impurities, and degradation products against the USP Reference Standard for the antibiotic. Establish values for relative microbial activity (*F*) for each moiety as compared with the USP Reference Standard. Active moieties, process impurities, and degradation products at levels below

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1% of antimicrobial activity may be disregarded. The candidate alternative method may resolve impurities that do not have antimicrobial activity. These will likely not factor into the bridging study.

2. Evaluate a suitable number of production lots of the antibiotic to determine whether the composition of the complex antibiotic is consistent from one lot to another.
3. Validate the candidate alternative procedure using ⟨1225⟩. The procedure must be specific, selective, and stability indicating.
4. Test a minimum of three separate lots of the drug substance and the USP Reference Standard using the microbial assay method and the candidate alternative procedure.
5. Test a minimum of six replicate samples per lot using both the candidate alternative method and the microbial assay procedures. It may be necessary to increase the number of replicates based on the maximum allowed percentage difference (see *Data Evaluation*) and the standard deviation of the method (*4*). If possible, prepare stock standard and sample solutions and subdivide them for use with the microbial assay and candidate alternative assay procedures. This provides paired data that can be analyzed (see *Appendix 2*).
6. Use the relative microbial activity (*F*) to convert the percentage purity values for each moiety, then sum them to determine a combined potency value.
7. Apply appropriate outlier (see ⟨1010⟩ and ⟨81⟩) and comparison tests (see *Data Evaluation*) to determine whether the candidate alternative procedure and the microbial assay procedure yield equivalent results.
8. If there are statistical outliers with either procedure, perform a comparison by excluding the outlier values. If the bridging study criteria are met, repeat the bridging study using controls to prevent outliers. Justification for rejection of outliers must be provided in the bridging study report. The consistent appearance of outliers in the candidate alternative assay results may indicate that the procedure has inadequate controls. If this occurs, the bridging study is void and a different or modified alternative procedure should be developed and validated.
9. If the bridging study fails, it may be necessary to include potency contributions from impurities that make a contribution below 1%.

DATA EVALUATION

Described below are USP's recommendations for data evaluation. Alternative approaches such as Deming regression (*5*) and commercially available comparison tests may also be used. For an antibiotic with a comparatively narrow Assay range (80%–125% or narrower), follow *Step 1*. For an antibiotic with a wide Assay range (80%–125% or wider), follow *Steps 1* and *2*.

1. Demonstrate the equivalence of results at the targeted potency of 100% using a two one-sided test (TOST) to test for equivalence. TOST offers several advantages over the *t*-test, which looks for differences (*6*,*7*,*8*,*9*). This requires the laboratory to set a maximum allowed percentage difference (such as 3%, 4%, or 5%), denoted by *k* in the appendices. Use the formulas in *Appendix 1* for independent samples (different samples used for the two procedures) or *Appendix 2* for paired samples (a set of samples each of which is assayed by both procedures).
2. Compare the chapter microbial assay and candidate alternative assay procedures using paired samples that cover the full monograph range of activity values. Prepare a Bland–Altman (BA) plot (*Appendix 3*). There should be no evidence of an important trend and the BA 95% agreement limits should not extend outside a predetermined maximum difference established by the laboratory.

APPENDIX 1: TOST FORMULAS FOR INDEPENDENT SAMPLES

On the basis of the knowledge of the product, the laboratory must establish the maximum allowed percentage difference between the average result for the candidate alternative assay and the average for the microbial assay results for the alternative assay. A difference that meets the requirements indicates that the alternative method provides acceptable results in the specified compendial range as compared with the microbial assay. In statistical notation, what must be demonstrated is:

$$100\left|\frac{\mu_{HPLC}}{\mu_{Micro}} - 1\right| < 100k \qquad \text{(Eq. 1)}$$

k = a small positive number such as 0.03 (for an allowed 3% difference)
μ_i = mean value for each procedure
i = alternative method (HPLC) or microbial assay (Micro)
These are the two values to be compared.
Rearranging, this becomes:

$$-k\mu_{Micro} < \mu_{HPLC} - \mu_{Micro} < k\mu_{Micro} \text{ (Eq. 2)}$$

The idea of TOST is to consider the two inequalities of *Equation 2* separately. That is, to demonstrate the hypothesis in *Equation 2* at the 5% level, one must demonstrate both of the inequalities in *Equation 3* at the 5% level:

$$\mu_{HPLC} - (1 + k)\mu_{Micro} < 0 \text{ and } \mu_{HPLC} - (1 - k)\mu_{Micro} > 0 \text{ (Eq. 3)}$$

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For a comparison of means, the TOST is equivalent to considering two-sided 90% confidence bounds. If the bounds satisfy the inequalities in *Equation 3*, then equivalence has been demonstrated. When the samples for the two assays under comparison are different ("independent samples"), determine the following upper (U) and lower (L) confidence bounds:

$$U = \overline{X}_{HPLC} - (1+k)\overline{X}_{Micro} + t_{0.05,df}\sqrt{S^2_{HPLC} / N_{HPLC} + (1+k)^2 S^2_{Micro} / N_{Micro}} \quad \text{(Eq. 4)}$$

$$L = \overline{X}_{HPLC} - (1-k)\overline{X}_{Micro} - t_{0.05,df}\sqrt{S^2_{HPLC} / N_{HPLC} + (1-k)^2 S^2_{Micro} / N_{Micro}} \quad \text{(Eq. 5)}$$

$\overline{X}_i$ = sample average
S_i = sample standard deviation
N = sample size
t = one-sided 5% value for a t-distribution
i = alternative method (HPLC) or microbial assay method (Micro)
For the number of degrees of freedom, use:

$$\frac{\left(\frac{s^2_{Micro}}{N_{Micro}} + \frac{s^2_{HPLC}}{N_{HPLC}}\right)^2}{\left[\left(\frac{1}{N_{Micro}-1}\right)\left(\frac{s^2_{Micro}}{N_{Micro}}\right)^2\right] + \left[\left(\frac{1}{N_{HPLC}-1}\right)\left(\frac{s^2_{HPLC}}{N_{HPLC}}\right)^2\right]} \quad \text{(Eq. 6)}$$

This is an approximation that assumes k is small. If using software that only allows for integer degrees of freedom (e.g., Excel), use linear interpolation to obtain the t-value. Conclude that the two procedures are equivalent (i.e., any difference on average is acceptably small) for the given lot if:

$$L > 0 \text{ and } U < 0 \quad \text{(Eq. 7)}$$

Example 1:

Microbial Assay Data	HPLC Assay Data
72.02	72.68
67.3	72.24
71.79	72.5
71.16	—
69.06	—
75.56	—
74.7	—
74.16	—
76.48	—

Following the above formulas with $k = 0.03$:

$$N_{HPLC} = 3;\ \overline{X}_{HPLC} = 72.5;\ S_{HPLC} = 0.221$$

$$N_{Micro} = 9;\ \overline{X}_{Micro} = 72.5;\ S_{Micro} = 3.045$$

Degrees of freedom = 8.247; interpolated t-value = 1.853

$L = 0.338$ and $U = -0.219$, so the 3% equivalence criterion is satisfied.

APPENDIX 2: TOST FORMULAS FOR PAIRED SAMPLES

When the samples for the two assays are the same, the data are considered "paired". The hypotheses are the same as those in *Appendix 1*. Because of the pairing of samples, the standard deviation calculations differ.
Determine the confidence bounds, U and L, as follows:

$$U = \overline{X}_{HPLC} - (1+k)\overline{X}_{Micro} + t_{0.05,df}\sqrt{S^2_U / N} \quad \text{(Eq. 8)}$$

$$L = \overline{X}_{HPLC} - (1-k)\overline{X}_{Micro} - t_{0.05,df}\sqrt{S^2_L / N} \quad \text{(Eq. 9)}$$

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N = number of samples for each procedure
$df = N - 1$
S_L and S_U are calculated as follows:

$$S_U^2 = \frac{1}{N-1}\sum_{j=1}^{N}[X_{HPLC,j} - (1+k)X_{Micro,j}]^2 \quad \text{(Eq. 10)}$$

$$S_L^2 = \frac{1}{N-1}\sum_{j=1}^{N}[X_{HPLC,j} - (1-k)X_{Micro,j}]^2 \quad \text{(Eq. 11)}$$

Conclude that the two procedures are equivalent (i.e., any difference on average is acceptably small) for the given lot if:

$$L > 0 \text{ and } U < 0 \text{ (Eq. 12)}$$

Example 2:

Microbial Assay Data	HPLC Assay Data
1011	980.9
990	981.4
960	978.3
1000	974.3
970	966.7

Following the above formulas with $k = 0.03$:

$$N = 5;\ \bar{X}_{HPLC} = 976.3;\ \bar{X}_{Micro} = 986.2$$

$$S_L = 18.749;\ S_U = 19.958$$

$$df = 4;\ t\text{-value} = 2.132$$

$L = 1.830$ and $U = -20.438$, so the 3% equivalence criterion is satisfied.

APPENDIX 3: BLAND–ALTMAN PLOTS

Figure 1 shows the data plotted as HPLC (x-axis) vs. Micro (y-axis). In *Figure 2*, the x-axis represents the average response obtained using the alternative and microbial assay methods.

The y-axis represents the difference in responses measured with the alternative and microbial assay methods.

The bias for each data set is represented by the average difference.

95% limits of agreement are represented by bias ± 2S.

Data points outside the limits of agreement are considered outliers.

The Bland–Altman plot (*10,11*) should show data points scattered within the limits of agreement with no obvious pattern. If a pattern exists (e.g., proportional pattern or funneling outwards with increasing average), perform a log transformation. If performing a log transformation, take the antilog of results for analysis using the original scale of measurement.

Example 3:

Level	Microbial Assay Data	HPLC Assay Data
150	854	867
150	862	893
150	871	880
150	845	906
150	836	854
120	678	704
120	699	722
120	688	739
120	705	686
120	671	669
90	530	541

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Level	Microbial Assay Data	HPLC Assay Data
90	536	554
90	522	528
90	510	515
90	515	502
60	366	343
60	363	361
60	348	334
60	352	370
60	357	352
30	197	185
30	187	172
30	192	176
30	189	180
30	195	167

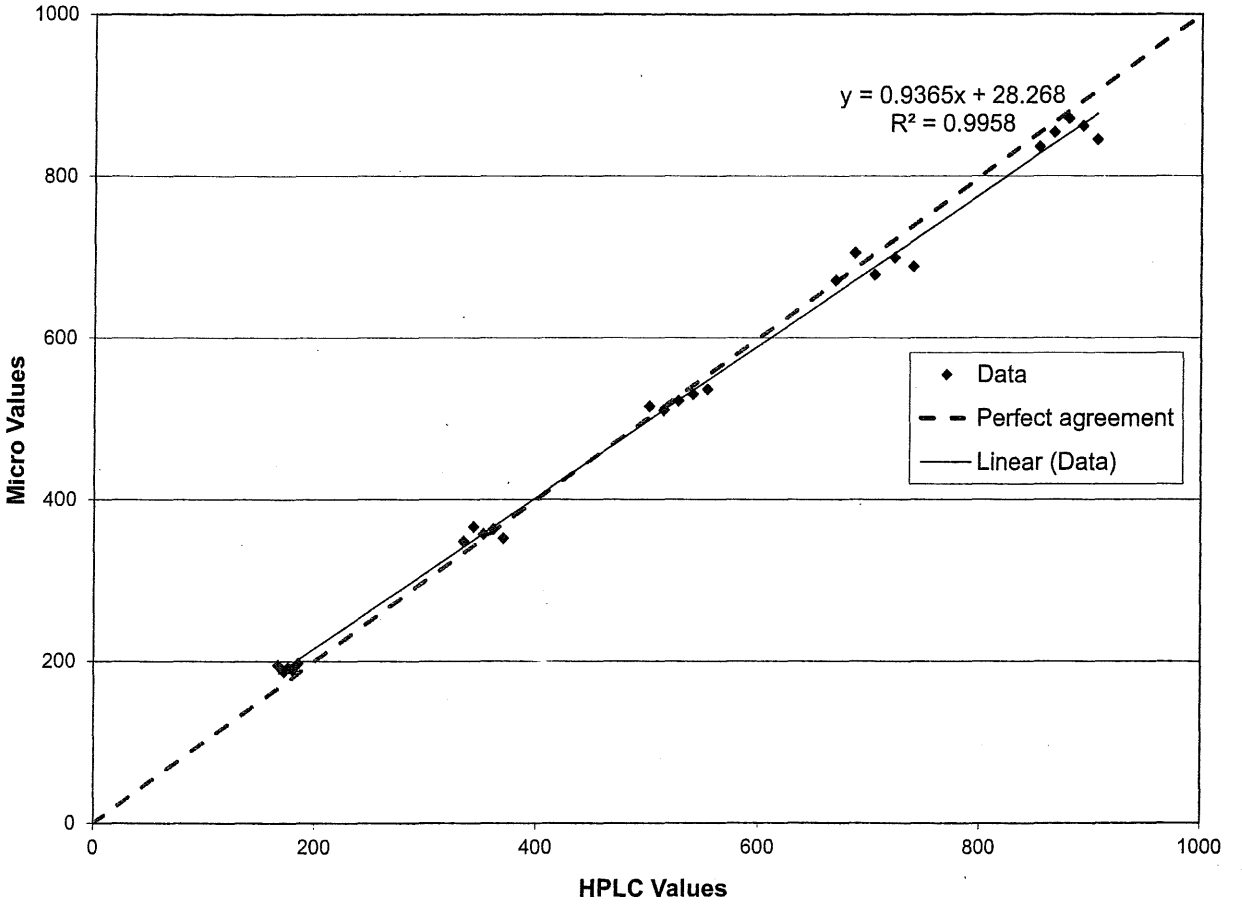

Figure 1. Example data plotted as Microbial assay data versus HPLC assay data.

Figure 1 shows the data plotted as Micro (*y*-axis) vs. HPLC (*x*-axis). This appears to be in good agreement. *Figure 2* shows what happens when the same data are instead plotted as recommended by Bland and Altman.

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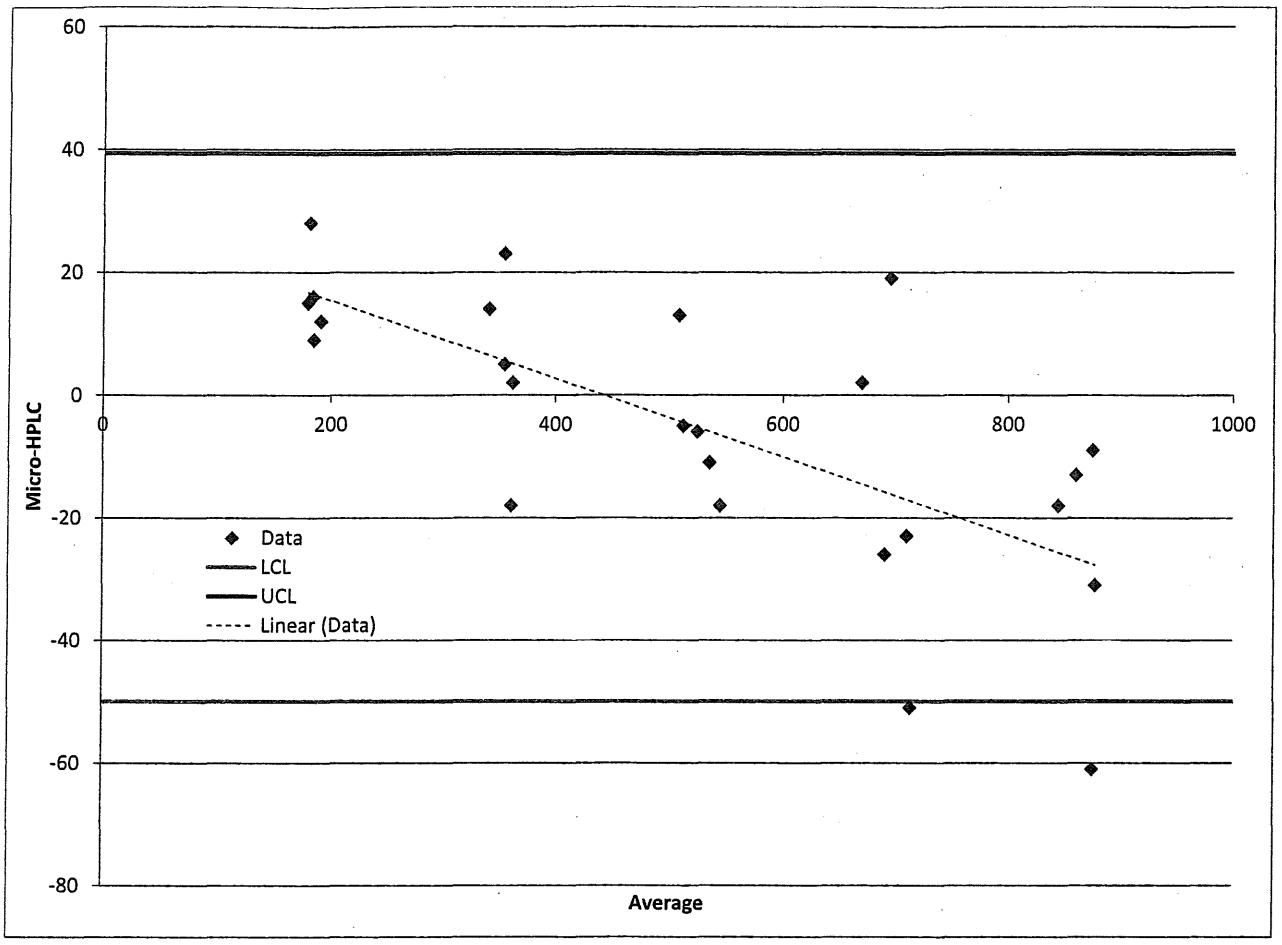

Figure 2. Example data in a Bland–Altman plot.

Calculate the bias, estimated by the mean difference (d) and standard deviation (SD) of the difference.

Mean difference = –5.28

SD of difference = 22.36

Most of the differences are expected to lie between d – 2SD and d + 2SD (if normally distributed, 95% will lie in these limits). If differences within d ± 2SD (limits of agreement) are not important, the two measurement methods can be used interchangeably.

LCL (d – 2SD) = –49.95 (Eq. 13)

UCL (d + 2SD) = 39.39 (Eq. 14)

The difference between results from the two procedures has a strong trend. This trend can be seen in *Figure 1*, but the Bland–Altman plot in *Figure 2* makes it clearer.

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⟨1224⟩ TRANSFER OF ANALYTICAL PROCEDURES

INTRODUCTION

Testing to the specification of an ancillary material, intermediate, and/or ingredient and product is critical in establishing the quality of a finished dosage form. The transfer of analytical procedures (TAP), also referred to as method transfer, is the documented process that qualifies a laboratory (the receiving unit) to use an analytical test procedure that originated in another laboratory (the transferring unit), thus ensuring that the receiving unit has the procedural knowledge and ability to perform the transferred analytical procedure as intended.

The purpose of this general information chapter is to summarize the types of transfers that may occur, including the possibility of waiver of any transfer, and to outline the potential components of a transfer protocol. The chapter does not provide statistical methods and does not encompass the transfer of microbiological or biological procedures.

TYPES OF TRANSFERS OF ANALYTICAL PROCEDURES

TAP can be performed and demonstrated by several approaches. The most common is comparative testing performed on homogeneous lots of the target material from standard production batches or samples intentionally prepared for the test (e.g., by spiking relevant accurate amounts of known impurities into samples). Other approaches include covalidation between laboratories, the complete or partial validation of the analytical procedures by the receiving unit, and the transfer waiver, which is an appropriately justified omission of the transfer process. The tests that will be transferred, the extent of the transfer activities, and the implementation strategy should be based on a risk analysis that considers the previous experience and knowledge of the receiving unit, the complexity and specifications of the product, and the procedure.

Comparative Testing

Comparative testing requires the analysis of a predetermined number of samples of the same lot by both the sending and the receiving units. Other approaches may be valid, e.g., if the receiving unit meets a predetermined acceptance criterion for the recovery of an impurity in a spiked product. Such analysis is based on a preapproved transfer protocol that stipulates the details of the procedure, the samples that will be used, and the predetermined acceptance criteria, including acceptable variability. Meeting the predetermined acceptance criteria is necessary to assure that the receiving unit is qualified to run the procedure.

Covalidation Between Two or More Laboratories

The laboratory that performs the validation of an analytical procedure is qualified to run the procedure. The transferring unit can involve the receiving unit in an interlaboratory covalidation, including them as a part of the validation team at the transferring unit and thereby obtaining data for the assessment of reproducibility. This assessment is made using a preapproved transfer or validation protocol that provides the details of the procedure, the samples to be used, and the predetermined acceptance criteria. The general chapter *Validation of Compendial Procedures* ⟨1225⟩ provides useful guidance about which characteristics are appropriate for testing.

Revalidation

Revalidation or partial revalidation is another acceptable approach for transfer of a validated procedure. Those characteristics described in ⟨1225⟩, which are anticipated to be affected by the transfer, should be addressed.

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Transfer Waiver

The conventional TAP may be omitted under certain circumstances. In such instances, the receiving unit is considered to be qualified to use the analytical test procedures without comparison and generation of interlaboratory comparative data. The following examples give some scenarios that may justify the waiver of TAP:

- The new product's composition is comparable to that of an existing product and/or the concentration of active ingredient is similar to that of an existing product and is analyzed by procedures with which the receiving unit already has experience.
- The analytical procedure being transferred is described in the *USP–NF*, and is unchanged. Verification should apply in this case (see ⟨1226⟩).
- The analytical procedure transferred is the same as or very similar to a procedure already in use.
- The personnel in charge of the development, validation, or routine analysis of the product at the transferring unit are moved to the receiving unit.

If eligible for transfer waiver, the receiving unit should document it with appropriate justifications.

ELEMENTS RECOMMENDED FOR THE TRANSFER OF ANAYTICAL PROCEDURES

Several elements, many of which may be interrelated, are recommended for a successful TAP. When appropriate and as a part of pretransfer activities, the transferring unit should provide training to the receiving unit, or the receiving unit should run the procedures and identify any issues that may need to be resolved before the transfer protocol is signed. Training should be documented.

The transferring unit, often the development unit, is responsible for providing the analytical procedure, the reference standards, the validation reports, and any necessary documents, as well as for providing the necessary training and assistance to the receiving unit as needed during the transfer. The receiving unit may be a quality control unit, another intracompany facility, or another company such as a contract research organization. The receiving unit provides qualified staff or properly trains the staff before the transfer, ensures that the facilities and instrumentation are properly calibrated and qualified as needed, and verifies that the laboratory systems are in compliance with applicable regulations and in-house general laboratory procedures. Both the transferring and receiving units should compare and discuss data as well as any deviations from the protocol. This discussion addresses any necessary corrections or updates to the final report and the analytical procedure as necessary to reproduce the procedure.

A single lot of the article may be used for the transfer, because the aim of the transfer is not related to the manufacturing process but rather to the evaluation of the analytical procedure's performance at the receiving site.

PREAPPROVED PROTOCOL

A well-designed protocol should be discussed, agreed upon, and documented before the implementation of TAP. The document expresses a consensus between the parties, indicating an intended execution strategy, and should include each party's requirements and responsibilities. It is recommended that the protocol contain the following topics as appropriate: objective, scope, responsibilities of the transferring and receiving units, materials and instruments that will be used, analytical procedure, experimental design, and acceptance criteria for all tests and/or methods included in the transfer. Based on the validation data and procedural knowledge, the transfer protocol should identify the specific analytical performance characteristics (see ⟨1225⟩ and ⟨1226⟩) that will be evaluated and the analysis that will be used to evaluate acceptable outcomes of the transfer exercise.

The transfer acceptance criteria, which are based on method performance and historical data from stability and release results, if available, should include the comparability criteria for results from all study sites. These criteria may be derived using statistical principles based on the difference between mean values and established ranges and should be accompanied by an estimation of the variability (e.g., percent relative standard deviation [%RSD] for each site), particularly for the intermediate precision %RSD of the receiving unit and/or a statistical method for the comparison of the means for assay and content uniformity tests. In instances of impurity testing, where precision may be poorer such as in the case of trace impurities, a simple descriptive approach can be used. Dissolution can be evaluated by a comparison of the dissolution profiles using the similarity factor f_2 or by comparison of data at the specified time points. The laboratories should provide appropriate rationale for any analytical performance characteristic not included. The materials, reference standards, samples, instruments, and instrumental parameters that will be used should be described.

It is recommended that expired, aged, or spiked samples be carefully chosen and evaluated to identify potential problems related to differences in sample preparation equipment and to evaluate the impact of potential aberrant results on marketed products. The documentation section of the transfer protocol may include report forms to ensure consistent recording of results and to improve consistency between laboratories. This section should contain the additional information that will be included with the results, such as example chromatograms and spectra, along with additional information in case of a deviation. The protocol should also explain how any deviation from the acceptance criteria will be managed. Any changes to the transfer protocol following failure of an acceptance criterion must be approved before collection of additional data.

THE ANALYTICAL PROCEDURE

The procedure should be written with sufficient detail and explicit instructions, so that a trained analyst can perform it without difficulty. A pretransfer meeting between the transferring and receiving units is helpful to clarify any issues and answer any questions regarding the transfer process. If complete or partial validation data exist, they should be available to the receiving

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unit, along with any technical details required to perform the test in question. In some cases it may be useful for the individuals who were involved with the initial development or validation to be on site during the transfer. The number of replicates and injection sequences in the case of liquid or gas chromatography should be clearly expressed, and, in the case of dissolution testing, the number of individual dosage units should be stipulated.

TRANSFER REPORT

When the TAP is successfully completed, the receiving unit should prepare a transfer report that describes the results obtained in relation to the acceptance criteria, along with conclusions that confirm that the receiving unit is now qualified to run the procedure. Any deviations should be thoroughly documented and justified. If the acceptance criteria are met, the TAP is successful and the receiving unit is qualified to run the procedure. Otherwise, the procedure cannot be considered transferred until effective remedial steps are adopted in order to meet the acceptance criteria. An investigation may provide guidance about the nature and extent of the remedial steps, which may vary from further training and clarification to more complex approaches, depending on the particular procedure.

⟨1225⟩ VALIDATION OF COMPENDIAL PROCEDURES

Test procedures for assessment of the quality levels of pharmaceutical articles are subject to various requirements. According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the *USP–NF* constitute legal standards. The Current Good Manufacturing Practice regulations [21 CFR 211.194(a)] require that test methods, which are used for assessing compliance of pharmaceutical articles with established specifications, must meet proper standards of accuracy and reliability. Also, according to these regulations [21 CFR 211.194(a)(2)], users of analytical methods described in *USP–NF* are not required to validate the accuracy and reliability of these methods, but merely verify their suitability under actual conditions of use. Recognizing the legal status of *USP* and *NF* standards, it is essential, therefore, that proposals for adoption of new or revised compendial analytical procedures be supported by sufficient laboratory data to document their validity.

The text of this information chapter harmonizes, to the extent possible, with the International Council for Harmonisation (ICH) tripartite guideline *Validation of Analytical Procedures* and the *Methodology* extension text, which are concerned with analytical procedures included as part of registration applications submitted within the EC, Japan, and the USA.

SUBMISSIONS TO THE COMPENDIA

Submissions to the compendia for new or revised analytical procedures should contain sufficient information to enable members of the USP Council of Experts and its Expert Committees to evaluate the relative merit of proposed procedures. In most cases, evaluations involve assessment of the clarity and completeness of the description of the analytical procedures, determination of the need for the procedures, and documentation that they have been appropriately validated. Information may vary depending upon the type of method involved. However, in most cases a submission will consist of the following sections.

Rationale

This section should identify the need for the procedure and describe the capability of the specific procedure proposed and why it is preferred over other types of determinations. For revised procedures, a comparison should be provided of limitations of the current compendial procedure and advantages offered by the proposed procedure.

Proposed Analytical Procedure

This section should contain a complete description of the analytical procedure sufficiently detailed to enable persons "skilled in the art" to replicate it. The write-up should include all important operational parameters and specific instructions such as preparation of reagents, performance of system suitability tests, description of blanks used, precautions, and explicit formulas for calculation of test results.

Data Elements

This section should provide thorough and complete documentation of the validation of the analytical procedure. It should include summaries of experimental data and calculations substantiating each of the applicable analytical performance characteristics. These characteristics are described in the following section.

VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Typical analytical performance characteristics that should be considered in the validation of the types of procedures described in this document are listed in *Table 1*. Because opinions may differ with respect to terminology and use, each of the performance characteristics is defined

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in the next section of this chapter, along with a delineation of a typical method or methods by which it may be measured. The definitions refer to "test results". The description of the analytical procedure should define what the test results for the procedure are. As noted in ISO 5725-1 and 3534-1, a test result is "the value of a characteristic obtained by carrying out a specified test method. The test method should specify that one or a number of individual measurements be made, and their average, or another appropriate function (such as the median or the standard deviation), be reported as the test result. It may also require standard corrections to be applied, such as correction of gas volumes to standard temperature and pressure. Thus, a test result can be a result calculated from several observed values. In the simple case, the test result is the observed value itself." A test result also can be, but need not be, the final, reportable value that would be compared to the acceptance criteria of a specification. Validation of physical property methods may involve the assessment of chemometric models. However, the typical analytical characteristics used in method validation can be applied to the methods derived from the use of the chemometric models.

Table 1. Typical Analytical Characteristics Used in Method Validation

Accuracy
Precision
Specificity
Detection limit
Quantitation limit
Linearity
Range
Robustness

The effects of processing conditions and potential for segregation of materials should be considered when obtaining a representative sample to be used for validation of procedures.

In the case of compendial procedures, revalidation may be necessary in the following cases: a submission to the USP of a revised analytical procedure or the use of an established general procedure with a new product or raw material (see below in *Data Elements Required for Validation*).

The ICH documents give guidance on the necessity for revalidation in the following circumstances: changes in the synthesis of the drug substance, changes in the composition of the drug product, and changes in the analytical procedure.

This chapter is intended to provide information that is appropriate to validate a wide range of compendial analytical procedures. The validation of compendial procedures may use some or all of the suggested typical analytical characteristics used in method validation as outlined in *Table 1* and categorized by type of analytical method in *Table 2*. For some compendial procedures the fundamental principles of validation may extend beyond characteristics suggested in this chapter. For these procedures the user is referred to the individual compendial chapter for those specific analytical validation characteristics and any specific validation requirements.

Analytical Performance Characteristics

ACCURACY

Definition: The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. [A note on terminology: The definition of accuracy in this chapter and ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), "accuracy" has a different meaning. In ISO, accuracy combines the concepts of unbiasedness (termed "trueness") and precision.]

Determination: In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., "to spike") or to compare results with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of quantitative analysis of impurities, accuracy should be assessed on samples (of drug substance or drug product) spiked with known amounts of impurities. Where it is not possible to obtain samples of certain impurities or degradation products, results should be compared with those obtained by an independent procedure. In the absence of other information, it may be necessary to calculate the amount of an impurity based on comparison of its response to that of the drug substance; the ratio of the responses of equal amounts of the impurity and the drug substance (relative response factor) should be used, if known.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual

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concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, or alternatively, that the slope be close to 1.0. In either case, the interval or the definition of closeness should be specified in the validation protocol. The acceptance criterion will depend on the assay and its variability and on the product. Setting an acceptance criterion based on the lack of statistical significance of the test of the null hypothesis that the slope is 1.0 is not an acceptable approach.

Accuracy of physical property methods may be assessed through the analysis of standard reference materials, or alternatively, the suitability of the above approaches may be considered on a case-by-case basis.

PRECISION

Definition: The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.
Determination: The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration.

SPECIFICITY

Definition: The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures. [NOTE—Other reputable international authorities (IUPAC, AOAC-I) have preferred the term "selectivity", reserving "specificity" for those procedures that are completely selective.] For the tests discussed below, the above definition has the following implications.

Identification tests: Ensure the identity of the analyte.

Purity tests: Ensure that all of the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., related substances test, heavy metals limit, or organic volatile impurities).

Assays: Provide an exact result, which allows an accurate statement on the content or potency of the analyte in a sample.
Determination: In the case of qualitative analyses (identification tests), the ability to select between compounds of closely related structure that are likely to be present should be demonstrated. This should be confirmed by obtaining positive results (perhaps by comparison to a known reference material) from samples containing the analyte, coupled with negative results from samples that do not contain the analyte and by confirming that a positive response is not obtained from materials structurally similar to or closely related to the analyte.

In the case of analytical procedures for impurities, specificity may be established by spiking the drug substance or product with appropriate levels of impurities and demonstrating that these impurities are determined with appropriate accuracy and precision.

In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure (e.g., a pharmacopeial or other validated procedure). These comparisons should include samples stored under relevant stress conditions (e.g., light, heat, humidity, acid/base hydrolysis, and oxidation). In the case of the assay, the results should be compared; in the case of chromatographic impurity tests, the impurity profiles should be compared.

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

For validation of specificity for qualitative and quantitative determinations by spectroscopic methods, chapters related to topics such as near-infrared spectrophotometry, Raman spectroscopy, and X-ray powder diffraction should be consulted.

DETECTION LIMIT

Definition: The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample.
Determination: For noninstrumental procedures, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

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For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. Rather, the detection limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the required detection level. For example, if it is required to detect an impurity at the level of 0.1%, it should be demonstrated that the procedure will reliably detect the impurity at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal-to-noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever method is used, the detection limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the detection limit.

QUANTITATION LIMIT

Definition: The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable *Precision* and *Accuracy* under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample.
Determination: For noninstrumental procedures, the quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable *Accuracy* and *Precision*.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the quantitation level. For example, if it is required that an analyte be assayed at the level of 0.1 mg/tablet, it should be demonstrated that the procedure will reliably quantitate the analyte at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal-to-noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever approach is used, the quantitation limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the quantitation limit.

LINEARITY AND RANGE

Definition of linearity: The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Thus, in this section, "linearity" refers to the linearity of the relationship of concentration and assay measurement. In some cases, to attain linearity, the concentration and/or the measurement may be transformed. [NOTE—The weighting factors used in the regression analysis may change when a transformation is applied.] Possible transformations may include log, square root, or reciprocal, although other transformations are acceptable. If linearity is not attainable, a nonlinear model may be used. The goal is to have a model, whether linear or nonlinear, that describes closely the concentration–response relationship.
Definition of range: The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent or parts per million) obtained by the analytical procedure.
Determination of linearity and range: Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, *y*-intercept, slope of the regression line, and residual sum of squares should be submitted.

The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered:

Assay of a drug substance (or a finished product): From 80% to 120% of the test concentration

Determination of an impurity: From 50% to 120% of the acceptance criterion

For content uniformity: A minimum of 70%–130% of the test concentration, unless a wider or more appropriate range based on the nature of the dosage form (e.g., metered-dose inhalers) is justified

For dissolution testing: ±20% over the specified range (e.g., if the acceptance criteria for a controlled-release product cover a region from 30% after 1 h, and up to 90% after 24 h, the validated range would be 10%–110% of the label claim).

The traditional definition of linearity, i.e., the establishment of a linear or mathematical relationship between sample concentration and response, is not applicable to particle size analysis. For particle size analysis, a concentration range is defined (instrument- and particle size-dependent) such that the measured particle size distribution is not affected by changes in concentration within the defined concentration range. Concentrations below the defined concentration range may introduce an error due to poor signal-to-noise ratio, and concentrations exceeding the defined concentration range may introduce an error due to multiple scattering.

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ROBUSTNESS

Definition: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

SYSTEM SUITABILITY

If measurements are susceptible to variations in analytical conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of *Robustness* and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used. Typical variations are the stability of analytical solutions, different equipment, and different analysts. In the case of liquid chromatography, typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. In the case of gas chromatography, typical variations are different lots or suppliers of columns, the temperature, and the flow rate.

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. They are especially important in the case of chromatographic procedures. Submissions to the USP should make note of the requirements in *Chromatography* ⟨621⟩, *System Suitability*.

Data Elements Required for Validation

Compendial test requirements vary from highly exacting analytical determinations to subjective evaluation of attributes. Considering this broad variety, it is only logical that different test procedures require different validation schemes. This chapter covers only the most common categories of tests for which validation data should be required. These categories are as follows:

CATEGORY I

Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

CATEGORY II

Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

CATEGORY III

Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, and others).

CATEGORY IV

Identification tests.

For each category, different analytical information is needed. Listed in *Table 2* are data elements that are normally required for each of these categories. [NOTE—For detailed information regarding the validation of dissolution procedures, see *The Dissolution Procedure: Development and Validation* ⟨1092⟩.]

Table 2. Data Elements Required for Validation

Analytical Performance Characteristics	Category I	Category II		Category III	Category IV
		Quantitative	Limit Tests		
Accuracy	Yes	Yes	[a]	[a]	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	[a]	Yes
Detection limit	No	No	Yes	[a]	No
Quantitation limit	No	Yes	No	[a]	No
Linearity	Yes	Yes	No	[a]	No
Range	Yes	Yes	[a]	[a]	No

[a] May be required, depending on the nature of the specific test.

Already established general procedures (e.g., titrimetric determination of water or bacterial endotoxins) should be verified to establish their suitability for use, such as their accuracy (and absence of possible interference) when used for a new product or raw material.

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When validating physical property methods, consider the same performance characteristics required for any analytical procedure. Evaluate use of the performance characteristics on a case-by-case basis, with the goal of determining that the procedure is suitable for its intended use. The specific acceptance criteria for each validation parameter should be consistent with the intended use of the method.

Physical methods may also be classified into the four validation categories. For example, validation of a quantitative spectroscopic method may involve evaluation of *Category I* or *Category II Analytical Performance Characteristics*, depending on the method requirements. Qualitative physical property measurements, such as particle size, surface area, bulk and tapped density, which could impact performance characteristics, often best fit in *Category III*. *Category IV Analytical Performance Characteristics* usually applies to validation of qualitative identification spectroscopic methods. However, the various techniques may be used for different purposes, and the specific use of the method and characteristics of the material being analyzed should be considered when definitively applying a category to a particular type of method.

The validity of an analytical procedure can be verified only by laboratory studies. Therefore, documentation of the successful completion of such studies is a basic requirement for determining whether a procedure is suitable for its intended application(s). Current compendial procedures are also subject to regulations that require demonstration of suitability under actual conditions of use (see *Verification of Compendial Procedures* ⟨1226⟩ for principles relative to the verification of compendial procedures). Appropriate documentation should accompany any proposal for new or revised compendial analytical procedures.

⟨1226⟩ VERIFICATION OF COMPENDIAL PROCEDURES

The intent of this chapter is to provide general information on the verification of compendial procedures that are being performed for the first time to yield acceptable results utilizing the personnel, equipment, and reagents available. This chapter is not intended for retroactive application to already successfully established laboratory procedures. *Validation of Compendial Procedures* ⟨1225⟩ provides general information on characteristics that should be considered for various test categories and on the documentation that should accompany analytical procedures submitted for inclusion in *USP–NF*. Verification consists of assessing selected analytical performance characteristics, such as those that are described in ⟨1225⟩, to generate appropriate, relevant data rather than repeating the validation process.

Users of compendial analytical procedures are not required to validate these procedures when first used in their laboratories, but documented evidence of suitability should be established under actual conditions of use. In the United States, this requirement is established in 21 CFR 211.194(a)(2) of the current Good Manufacturing Practice regulations, which states that the "suitability of all testing methods used shall be verified under actual conditions of use."

Verification of microbiological procedures is not covered in this chapter because it is covered in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Microorganisms* ⟨62⟩, *Sterility Tests* ⟨71⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

Change to read:

VERIFICATION PROCESS

The verification process for compendial test procedures is the assessment of whether the procedure can be used for its intended purpose, under the actual conditions of use for a specified drug substance and/or drug product matrix.

Users should have the appropriate experience, knowledge, and training to understand and be able to perform the compendial procedures as written. Verification should be conducted by the user such that the results will provide confidence that the compendial procedure will perform suitably as intended.

If the verification of the compendial procedure is not successful, and assistance from USP staff has not resolved the problem, it may be concluded that the procedure may not be suitable for use with the article being tested in that laboratory. It may then be necessary to develop and validate an ▲alternative▲ (USP 1-Dec-2019) procedure as allowed in the *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*. The ▲alternative▲ (USP 1-Dec-2019) procedure may be submitted to USP, along with the appropriate data, to support a proposal for inclusion or replacement of the current compendial procedure.

Change to read:

VERIFICATION REQUIREMENTS

Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Although complete revalidation of a compendial method is not required to verify the suitability of a procedure under actual conditions of use, some of the analytical performance characteristics listed in *Validation of Compendial Procedures* ⟨1225⟩, *Table 2*, may be used for the verification process. Only those characteristics that are considered to be appropriate for the verification of the particular procedure need to be evaluated. The process of assessing the suitability of a compendial analytical test procedure under the conditions of actual use may or may not require actual laboratory performance of each analytical performance characteristic. The degree and extent of the verification process may depend on the level of training and experience of the user, on the type of procedure and its associated equipment or instrumentation, on the specific procedural steps, and on which article(s) are being tested.

Verification should assess whether the compendial procedure is suitable for the drug substance and/or the drug product matrix, taking into account the drug substance's synthetic route, the method of manufacture for the drug product, or both, if applicable. Verification should include an assessment of elements such as the effect of the matrix on the recovery of impurities

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and drug substances from the drug product matrix, as well as the suitability of chromatographic conditions and column, the appropriateness of detector signal response, etc.

As an example, an assessment of specificity is a key parameter in verifying that a compendial procedure is suitable for use in assaying drug substances and drug products. For instance, acceptable specificity for a chromatographic method may be verified by conformance with system suitability resolution requirements (if specified in the procedure). However, drug substances from different suppliers may have different impurity profiles that are not addressed by the compendial test procedure. Similarly, the excipients in a drug product can vary widely among manufacturers and may have the potential to directly interfere with the procedure or cause the formation of impurities that are not addressed by the compendial procedure. In addition, drug products containing different excipients, antioxidants, buffers, or container extractives may affect the recovery of the drug substance from the matrix. In these cases, a more thorough assessment of the matrix effects may be required to demonstrate suitability of the procedure for the particular drug substance or product. Other analytical performance characteristics such as an assessment of the limit of detection or quantitation and precision for impurities procedures may be useful to demonstrate the suitability of the compendial procedure under actual conditions of use. ▲It is the user's responsibility to demonstrate the long term (more than 24 h) stability and storage conditions of Standard and sample preparations throughout the duration of the procedure during the verification of compendial procedures.▲ (USP 1-Dec-2019)

Verification is not required for basic compendial test procedures that are routinely performed unless there is an indication that the compendial procedure is not appropriate for the article under test. Examples of basic compendial procedures include, but are not limited to, loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental determinations such as pH measurements. However, for the application of already established routine procedures to compendial articles tested for the first time, it is recommended that consideration be given to any new or different sample handling or solution preparation requirements.

⟨1227⟩ VALIDATION OF MICROBIAL RECOVERY FROM PHARMACOPEIAL ARTICLES

Change to read:

INTRODUCTION

▲This chapter provides guidelines for the validation of recovery methods for the estimation of the number of viable microorganisms, the detection of indicators or specified microorganisms, and the sterility testing of pharmacopeial articles. The test procedures in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Sterility Tests* ⟨71⟩, *Microbial Enumeration Tests* ⟨61⟩, and *Tests for Specified Microorganisms* ⟨62⟩ are considered validated. However, use of compendial methods requires establishment of suitability of the method demonstrating recovery of the challenge organisms in the presence of the product. Alternatives/modifications to these recovery procedures beyond what are described in these chapters (such as dilution, chemical or enzymatic neutralization, and membrane filtration) require validation.▲ (USP 1-Dec-2019) It is generally understood that if a product possesses antimicrobial properties because of the presence of a specific preservative or because of its formulation, this antimicrobial property must be neutralized to recover viable microorganisms. This neutralization may be achieved by the use of a specific neutralizer, by dilution, by a combination of ▲▲ (USP 1-Dec-2019) dilution, ▲filtration, and rinsing,▲ (USP 1-Dec-2019) or by any combination of these methods. ▲When the product displays intrinsic antimicrobial activity for a given microorganism and, given this antimicrobial activity, the risk of microbial contamination is low, the method could be considered as fit for the purpose of providing a strong rationale.▲ (USP 1-Dec-2019)

Change to read:

INFLUENTIAL FACTORS

Several factors affect the measurement of a test solution's antimicrobial activity, and these must be considered in the validation design. They include the nature of the microorganisms used as challenge organisms, preparation of the inoculum of challenge organisms, specific conditions of the test, and conditions of recovery. These factors also affect the validation of recovery methods for aqueous or nonaqueous products, irrespective of their antimicrobial properties; thus, all test methods should be validated with these factors in mind.

The nature of the challenge microorganism exerts a strong effect upon the response to the antimicrobial agent, and so upon the neutralization required for recovery. Represented among these organisms in compendial tests are gram-positive bacteria, gram-negative bacteria, ▲anaerobic bacteria,▲ (USP 1-Dec-2019) yeasts, and molds. Each organism to be used in the test must be included in the validation.

The preparation of the inoculum of challenge microorganisms also affects the testing of products having antimicrobial properties. The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any test of antimicrobial efficacy. Microbial tests do not use individual cells; rather, populations of cells are harvested for study. The data generated from these studies are less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for reproducible culture preparation. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and should reflect the conditions of the antimicrobial assay.

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The specific conditions of the test, including buffers used, water, light conditions, and temperature, must be reproduced in the validation study. All test conditions also should be standardized and performed in the validation study exactly as performed in the test.

The conditions of microbial recovery are among the most crucial in accurately estimating the number of microorganisms present in a test solution. The first consideration is the recovery medium used to support the growth of survivors. This concern is discussed in detail below. The second consideration is the incubation conditions. Optimal conditions for growth must be present to ensure complete growth and reproducible results.

Change to read:

METHODS OF NEUTRALIZING ANTIMICROBIAL PROPERTIES

Three common methods are used to neutralize antimicrobial properties of a product: 1) chemical inhibition, 2) dilution, and 3) filtration and ▲rinsing.▲ (USP 1-Dec-2019)

Chemical ▲Neutralization▲ (USP 1-Dec-2019)

Table 1 shows known neutralizers for a variety of chemical antimicrobial agents and the reported toxicity of some chemical neutralizers to specific microorganisms. However, despite potential toxicity, the convenience and quick action of chemical inhibitors encourage their use. Chemical ▲neutralization of antimicrobial agents▲ (USP 1-Dec-2019) is the preferred method for the antimicrobial efficacy test. The potential of chemical ▲neutralizers▲ (USP 1-Dec-2019) should be considered in the membrane filtration and the direct ▲inoculation▲ (USP 1-Dec-2019) sterility tests. Antibiotics may not be susceptible to neutralization by chemical means, but rather by enzymatic treatment (e.g., penicillinase). These enzymes may be used where required.

Table 1. Some Common Neutralizers for Chemical ▲Antimicrobial Agents▲ (USP 1-Dec-2019)

Neutralizer	▲Antimicrobial▲ (USP 1-Dec-2019) Class	Potential Action of ▲Antimicrobial Agents▲ (USP 1-Dec-2019)
Bisulfate	Glutaraldehyde, mercurials	Non-sporing bacteria
Dilution	Phenolics, alcohol, aldehydes, sorbate	—
Glycine	Aldehydes	Growing cells
Lecithin	Quaternary ammonium compounds (QACs), parabens, bis-biguanides	Bacteria
Mg^{+2} or Ca^{+2} ions	EDTA	—
Polysorbate	QACS, iodine, parabens	—
Thioglycollate	Mercurials	Staphylococci and spores
Thiosulfate	Mercurials, halogens, aldehydes	Staphylococci

Dilution

A second approach to neutralizing antimicrobial properties of a product is by dilution, because the concentration of a chemical ▲antimicrobial agent▲ (USP 1-Dec-2019) exerts a large effect on its potency. The relationship between concentration and antimicrobial effect differs among bactericidal agents but is constant for a particular antimicrobial agent. This relationship is exponential in nature, with the general formula:

$$C^{\eta}t = k$$

C = concentration ▲of the antimicrobial agent▲ (USP 1-Dec-2019)
η = concentration exponent ▲(dilution coefficient),▲ (USP 1-Dec-2019) the slope of the plot of log t versus log C
t = time required to kill a standard inoculum
k = a constant

Antimicrobial agents with high η values are rapidly neutralized by dilution, whereas those with low η values are not good candidates for neutralization by dilution ▲(see *Table 2*).

Table 2. Concentration Exponents for Some Common Antimicrobial Agents

Representative Antimicrobial Agent	η Values	Increased Time Factor (x) to Kill Microorganisms When the Concentration Is Reduced to:	
		One-Half	One-Third
Phenolics	6	64	729
Alcohol	10	1024	59,000
Parabens	2.5	6	16

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Table 2. Concentration Exponents for Some Common Antimicrobial Agents *(continued)*

Representative Antimicrobial Agent	η Values	Increased Time Factor (*x*) to Kill Microorganisms When the Concentration Is Reduced to:	
		One-Half	One-Third
Chlorhexidine	2	4	8
Mercury compounds	1	2	3
Quaternary ammonium compounds	1	2	3
Formaldehyde	1	2	3▲ (USP 1-Dec-2019)

Membrane Filtration

An approach that is often used, especially in sterility testing, is neutralization by membrane filtration. This approach relies upon the physical retention of the microorganism on the membrane filter, with the antimicrobial agent passing through the filter into the filtrate. The filter is then incubated for recovery of viable microorganisms. However, filtration alone may not remove sufficient quantities of the ▲antimicrobial▲ (USP 1-Dec-2019) agent to allow growth of surviving microorganisms. Adherence of residual antimicrobial agents to the filter membrane may cause growth inhibition. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps to minimize this growth inhibition. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a ▲non-toxic▲ (USP 1-Dec-2019) fluid, such as diluting *Fluid A* (see *Sterility Tests* ⟨71⟩, *Diluting and Rinsing Fluids for Membrane Filtration* for diluting fluid compositions). Chemical neutralizers in the rinsing fluid can ensure that any antimicrobial residue on the membrane does not interfere with the recovery of viable microorganisms.

Change to read:

VALIDATION OF NEUTRALIZATION METHODS—RECOVERY COMPARISONS

A validated method for neutralizing the antimicrobial properties of a product must meet two criteria: neutralizer efficacy and neutralizer ▲non-toxicity.▲ (USP 1-Dec-2019) The validation study documents that the neutralization method employed is effective in inhibiting the antimicrobial properties of the product (neutralizer efficacy) without impairing the recovery of viable microorganisms (▲lack of▲ (USP 1-Dec-2019) neutralizer toxicity). Validation protocols may meet these two criteria by comparing recovery results for treatment groups.

The first is the test group, in which the product is subjected to the neutralization method, then a low level of challenge microorganism [less than 100 colony-forming units (cfu)] is inoculated for recovery. The second is the peptone control group, in which the neutralization method is used with peptone, or diluting *Fluid A* (see *Sterility Tests* ⟨71⟩, *Diluting and Rinsing Fluids for Membrane Filtration*), as the test solution. The third is the viability group, in which the actual inoculum is used without exposure to the neutralization scheme. Similar recovery between the test group and the peptone group demonstrates adequate neutralizer efficacy; similar recovery between the peptone group and the viability group demonstrates adequate neutralizer ▲non-toxicity.▲ (USP 1-Dec-2019)

In principle, the protocol must show that recovery of a low inoculum (less than 100 cfu) is not inhibited by the test sample and the neutralization method. Validation protocols may meet these two criteria by comparing recovery among three distinct test groups: 1) neutralized product with inoculum, 2) challenge inoculum control in buffered solution, and 3) inoculum in the absence of product or neutralizer. This can be established by directly comparing the result in the treated solution (1) to the inoculum (3) above. If the growth on the treated solution is not comparable to the growth on the inoculum group, it should be determined whether the neutralization method itself is toxic to the microorganisms.

Recovery on Agar Medium

In the tests under ⟨51⟩ and ⟨61⟩, ▲▲ (USP 1-Dec-2019)the number of viable challenge microorganisms in the product is estimated ▲▲ (USP 1-Dec-2019) by calculating the concentration of cfu per milliliter by the plate count method. A design for validating neutralization would incorporate the treatment groups as described under *Validation of Neutralization Methods—Recovery Comparisons*. At least three independent replicates of the experiment should be performed, and each should demonstrate ▲a mean count of any of the test organisms not differing by a factor greater than 2, i.e., 50%–200% recovery, from the value of the control in the absence of product. If it is necessary to solubilize the test stample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.▲ (USP 1-Dec-2019)

If a greater number of replicates is required in the validation study, the comparisons may be evaluated by transforming the numbers of cfu to their logarithmic values and analyzing the data statistically by the Student *t* test (pairwise comparisons) or by analysis of variance (ANOVA) (for comparing all groups). If ANOVA is used, and significant differences among the populations are determined, a test such as Dunnett's test may be used, with the peptone group used as the control group.

Recovery by Membrane Filtration

This validation follows the procedure described in ▲*Sterility Tests* ⟨71⟩, *Method Suitability Test*,▲ (USP 1-Dec-2019)with the exception of plating on solid medium to quantitate recovery. ▲It should be emphasized that quantitative recovery is not required to

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demonstrate sterility test suitability. It only requires a qualitative assessment (visual turbidity). ▲(USP 1-Dec-2019) Three 100-mL rinses are assumed, but the volume and number of rinses are subject to validation. ▲A maximum of five 100-mL washes should be used for routine testing even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity.▲ (USP 1-Dec-2019)Each validation run should be performed independently at least three times.

In the test solution group, the product is passed through the membrane filter, followed by two 100-mL portions of diluting-neutralizing fluid. After the second rinse has been filtered, a final 100-mL portion containing less than 100 cfu of the specific challenge microorganism is passed through the filter. This filter is then placed on the appropriate agar recovery medium and incubated for recovery.

The inoculum is directly plated onto the solid medium. It is possible that filtration will lead to reduced recovery of the challenge microorganism, either through inherent toxicity of the membrane or by adherence of the microorganism to the filtration vessel walls. A control group can be used to evaluate this component of membrane filtration validation. Diluting *Fluid A* is used as the dilution medium without exposing the filter to the product. After addition of the low-level inoculum to the final rinse, the filter is plated as above. Technique-specific loss of microorganisms can be estimated by comparing the recovery in the diluting *Fluid A* (see *Sterility Tests* ⟨71⟩, *Diluting and Rinsing Fluids for Membrane Filtration*) group to the inoculum count.

It is assumed in this discussion that the test sample can be filtered. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

The method can be considered validated if the recovery rate in the three independent replicates is similar for the test solution and the diluting *Fluid A* (see *Sterility Tests* ⟨71⟩, *Diluting and Rinsing Fluids for Membrane Filtration*) control.

Recovery in Liquid Medium

It is assumed in *Sterility Tests* ⟨71⟩, *Test for Sterility of the Product to be Examined, Direct Inoculation of the Culture Medium* that the recovery medium will allow for growth of all surviving microorganisms. The broth in that test must serve both to neutralize any antimicrobial properties of the test solution and to support the growth of the microorganisms. The treatment groups described under *Validation of Neutralization Methods—Recovery Comparisons* can be used for validation of the recovery method, with the proportions of product and recovery medium varied to achieve adequate neutralization. The method can be considered validated if all groups show ▲clearly visible growth visually comparable to that in the control vessel without product within the indicated time period in ⟨71⟩.▲ (USP 1-Dec-2019)

RECOVERY OF INJURED MICROORGANISMS

The validation studies described above use challenge microorganisms that have never been exposed to antimicrobial agents, and thus are not identical to organisms seen in antimicrobial effectiveness testing or when a sterility test is performed on a preserved product. If the use of alternative media is desired, the recovery of injured microorganisms should be addressed in the validation study. This may be done by directly comparing the recovery of each challenge microorganism on the preferred medium and on the alternative medium, after exposure to the product. This exposure should include at least two time periods showing survival of less than 100 cfu/mL, unless the rate of kill of the antimicrobial agent is such that no recovery is possible even if the microorganism is plated within minutes of exposure. This comparison should be performed at least three times. The alternative medium is validated if the recovery seen on that medium is no less than that seen on the preferred medium, within an error of 0.5 log units.

Change to read:

ESTIMATING THE NUMBER OF COLONY-FORMING UNITS

The accuracy of any estimate of viable cfu is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and *Candida albicans*. This range was established in the food industry for counting coliform bacteria in milk. This range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental isolates. The recommended counting range for *Aspergillus* ▲*brasiliensis*▲ (USP 1-Dec-2019) is between 8 and 80 cfu/plate.

▲▲ (USP 1-Dec-2019)

Lower counting thresholds for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson distribution, so the variance of the mean value equals the mean value of counts. Therefore, as the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see ▲*Table 3*). For example,▲ (USP 1-Dec-2019) 3 cfu/plate at the 10^{-1} dilution provide an estimate of 30 cfu/mL, with an error of 58% of the estimate.

▲Table 3.▲ (USP 1-Dec-2019) Error as a Percentage of Mean for Plate Counts

cfu/Plate	Standard Error	Error as % of Mean
30	5.48	18.3
29	5.39	18.6
28	5.29	18.9

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▲Table 3.▲ (USP 1-Dec-2019) Error as a Percentage of Mean for Plate Counts *(continued)*

cfu/Plate	Standard Error	Error as % of Mean
27	5.20	19.2
26	5.10	19.6
25	5.00	20.0
24	4.90	20.4
23	4.80	20.9
22	4.69	21.3
21	4.58	21.8
20	4.47	22.4
19	4.36	22.9
18	4.24	23.6
17	4.12	24.3
16	4.00	25.0
15	3.87	25.8
14	3.74	26.7
13	3.61	27.7
12	3.46	28.9
11	3.32	30.2
10	3.16	31.6
9	3.00	33.3
8	2.83	35.4
7	2.65	37.8
6	2.45	40.8
5	2.24	44.7
4	2.00	50.0
3	1.73	57.7
2	1.41	70.7
1	1.00	100.0

⟨1228⟩ DEPYROGENATION

INTRODUCTION

The production of parenteral products requires not only that products be sterile, but that they are also free from harmful levels of pyrogens. Depyrogenation is defined as the direct and validated destruction or removal of pyrogens. For the purposes of this and subsequent chapters of the ⟨1228⟩ series, the term "depyrogenation" refers to the destruction or removal of bacterial endotoxins, the most prevalent and quantifiable pyrogen in parenteral preparations. The chapters in this series discuss depyrogenation procedures that are applicable to product streams, equipment, and drug product containers and closures.

The ⟨1228⟩ series builds on the tenets described in *Sterility Assurance* ⟨1211⟩, first published in *USP 20–NF 15*. In the time since that publication, the science of depyrogenation has advanced, as has control over manufacturing processes. Manufacturers are very aware of the impact that facility, equipment, and process design has on endotoxin levels and depyrogenation, and they are mindful of the effects of elevated endotoxin levels in raw materials and water systems. Risk management tools, including process evaluation tools such as Hazard Analysis Critical Control Point (HACCP), are helping companies to identify critical control points (CCP) for the control of endotoxin and other impurities or contaminants in the manufacturing process. As a result of these advances in science, technology, and philosophy, the ⟨1228⟩ series will also offer alternatives to historical or traditional thinking.

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BACTERIAL ENDOTOXIN AND LIPOPOLYSACCHARIDE

Bacterial endotoxin is a component of the outer cell membrane of Gram-negative bacteria. The natural endotoxin complex contains many cell wall components including phospholipids, lipoproteins, and lipopolysaccharide (LPS), which is the biologically active component of endotoxin. Purified endotoxin is chemically defined as a LPS. LPS consists of three distinct regions:

1. The hydrophobic lipid A portion of the molecule is highly conserved among Gram-negative bacteria, and is largely responsible for most, if not all, of the biological activity of endotoxin.
2. A core oligosaccharide links the lipid A to the hydrophilic *O*-specific side chain or *O*-antigen.
3. The hydrophilic *O*-antigen is a highly variable region that confers serological specificity to the organism and is often used to distinguish strains of Gram-negative bacteria.

Due to the amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a nonpolar (hydrophobic) end], purified LPS preparations, such as reference standard endotoxin (RSE) and control standard endotoxin (CSE) tend to form bilayers, micelles, ribbons, and other conformations when in solution, and they may adsorb, or "stick", to surfaces, making them difficult to extract and detect. The degree of adsorption of LPS to solid surfaces is affected by the composition and finish of the material to be depyrogenated. The extent of aggregation of LPS in solution is affected by a host of formulation matrix attributes to which it is exposed, such as temperature, pH, salt concentration, divalent cation concentration, detergents or emulsifiers, and chelating agents.

When parenteral products are contaminated with endotoxin, the contaminant is not purified LPS, but rather whole cells or cell wall fragments generated during the normal growth cycle of the bacteria or disruption of bacteria, where the LPS remains embedded in or associated with other cell wall components. Purified LPS and native endotoxin are dissimilar in many respects, and the two terms should not be used interchangeably. Depending on the materials of construction or the formulation of the article to be depyrogenated, the use of native endotoxin as a challenge material in depyrogenation studies may be a consideration because a native endotoxin preparation better reflects operational reality, particularly for the depyrogenation of product streams, and because LPS molecules in natural endotoxin are embedded in cell wall complexes, they may be much less prone to the aggregation and adsorption issues seen with purified LPS.

For the purposes of the ⟨1228⟩ series, the term "challenge material" will be used to generically describe material (endotoxin or LPS) used as a spiking analyte for depyrogenation studies. "Endotoxin" will refer to the moiety in its natural state, meaning pieces of Gram-negative cell wall from a well-characterized source. LPS will refer to the purified material.

MEASURING ENDOTOXIN PRE- AND POST-PROCESSING

The primary procedure used for the measurement of bacterial endotoxin is the *Bacterial Endotoxins Test* ⟨85⟩ (BET). A well-controlled BET assay can provide assurance of accurate readings for the calculation of the reduction in challenge material activity pre- and post-processing, as well as provide consistent quantitation of levels of native endotoxin in raw materials, at CCP in the manufacturing process, and in finished products.

There are many variables in study structure and test method that can affect the outcome of a depyrogenation study. Development of a test method depends on the material under test, the identification of an appropriate challenge preparation, and the method used to extract recoverable activity prior to processing and residual activity after processing. Once a test system is developed that includes the identification of a source for challenge material preparations, inoculation of the articles to be depyrogenated (including drying procedures), extraction or recovery methods, and appropriate BET test methodology and sensitivity, it is recommended that subsequent tests should use the same conditions to ensure the comparability of test results. Points to consider when constructing a depyrogenation study include the following:

1. **The challenge material**: Consider the source of the endotoxin (purified or natural). When using purified LPS, choose a preparation with no fillers, because the presence of these fillers can add to the variability and therefore decrease the accuracy of the assay. Once challenge material preparation is chosen, it is recommended to use material from the same source in subsequent studies to reduce variability.[1]
2. **The characteristics of the material being depyrogenated**: It is important to understand the characteristics of the material being tested. For example, LPS may adsorb to plastics, and although two objects may be made of the same plastic, surface finish, surface area, and conformation differences may affect extraction efficiency and LPS recovery. For solutions, formulation matrices may affect aggregation of purified LPS in that pH, salt concentration, chelating agents, surfactants, and the presence of divalent cations may all have an impact on the recovery of the challenge material. The use of natural endotoxin may mitigate some of these recovery issues. For materials that are received with low or undetectable levels of endotoxin, depyrogenation studies using endotoxin or LPS challenge materials may be unnecessary if control is demonstrated and decisions are scientifically justified.
3. **The level of activity needed for the study**: How much pre-processing activity do you need to execute the study? The current industry standard is to add enough endotoxin to the system so that at least 1000 EU can be recovered prior to depyrogenation. However, depending on the test system, 1000 EU may be either excessive or insufficient. For example, when designing a study for the depyrogenation of a product stream that normally contains <1 EU in a certain volume, a spike of 1000 EU of a CSE in that same volume may be excessive. For solid-surface materials, the level of activity in the challenge material should be established taking into account the materials of construction and finish as they may contribute to LPS adsorption. Knowledge of historical levels of endotoxin in or on the surface, the efficiency of the depyrogenation processes, the efficiency of the challenge material extraction or recovery method, and the log reduction or safety level target acceptance criterion are all important to the setting of a pre-processing activity requirement. If a reduction study for a product stream is required, it may be more appropriate to add an amount of naturally occurring

[1] Ludwig JD, Avis KE. Recovery of endotoxin preparations from the surface of glass capillary tubes. *J Parenter Sci Technol.* 1989;43(6):276–278.

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endotoxin to the product consistent with the maximum expected endotoxin load ("worst case"), based on known endotoxin contributors (e.g., raw materials and water) and process capability to demonstrate reduction to safe levels. Whatever the procedure, the logic and methodology for endotoxin reduction studies should be justified and documented. For those materials that routinely contain a level of endotoxin, such as fermentation broths where there are high levels of activity to begin with, it may not be necessary to add additional challenge material.

4. **Preparation of test samples**: The method used to affix challenge materials to the surface of materials to be depyrogenated may affect its removal or recovery. Air-drying is the most convenient method of affixing challenge material to hard surfaces, but freeze drying and vacuum drying also have been used. To improve drying efficiency, it is suggested that a small volume of a highly concentrated activity of challenge material be used. This volume may be added to the surface of the item in an area that has been defined as "hardest to depyrogenate" or may be dispersed to represent the more likely natural occurrence. Inoculation methodology must be well defined for comparability across studies. Decisions regarding the design of studies must be documented and justified.
5. **Recovery methods**: Although there are standard methods for the recovery of endotoxin from medical devices, there is no standard method for the recovery of endotoxin dried onto solid surfaces that are used as indicators in depyrogenation studies. Although it is most convenient to adopt the standard extraction methods described in *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩ and ANSI/AAMI ST72:2011,[2] a laboratory may choose to develop and validate a method that better suits the material under test. The efficiency of the recovery of endotoxin will depend on the attributes of the material under test, the composition of the challenge material, the concentration of the challenge material spike, and the method of drying. Recoveries of less than 100% of the challenge material nominal spike in positive controls are not uncommon. Perhaps more important than the percent recovery in positive controls is consistent recovery across lots of the same material and across depyrogenation studies.[3, 4]
6. **Test method**: Lysate formulations differ, and they may be subject to different interferences such as leachables, chelators, and salts. If interferences are encountered during method development that are difficult to overcome, consider trying another test method (e.g., gel clot, kinetic chromogenic, kinetic turbidimetric, endpoint chromogenic) or a different source of reagent.
7. **Depyrogenation method**: Variability in the depyrogenation treatment may impact recovery. Treatments vary in efficiency, both within and between treatment types. For example, a large difference in efficiency will be seen when comparing the depyrogenation of glass vials using dry heat versus rinsing in water for injection (WFI), and differences may be seen in the reduction of challenge material by the filtration of a solution depending on the type of filter chosen. Likewise, variability can be seen when using a single depyrogenation methodology with two different materials, such as the filtration of two different solutions using the same type of filter medium.

The efficiency of a depyrogenation process has been historically measured in terms of a logarithmic (log) reduction of a large "spike" or bolus of purified endotoxin that is added to or dried onto a material prior to treatment. Although log reduction is a convenient measurement benchmark, the more relevant and pragmatic indicator of depyrogenation efficiency is one based on process capability and patient safety, which is the reduction of the measured or anticipated worst case natural levels of contaminating endotoxin to safe levels as defined by reference to calculated endotoxin limits for the material.

With the implementation of the principles of quality by design (QbD) and risk management, the 3-log reduction that was first introduced in 1984 may be inappropriate as a universal benchmark for modern depyrogenation processes. For example, materials with a high level of native endotoxin, such as fermentation broths, could require more than a 3-log reduction to reach a safe level, whereas dry heat depyrogenation of materials with normally low or undetected levels of native endotoxin, such as washed glass vials, will require substantially less than a 3-log reduction. The appropriate endotoxin log reduction for the process should be determined by the user based on a full understanding of the product and process capability including input sources, levels of endotoxin, efficiency of depyrogenation methods, and output (product- or process-specific) endotoxin requirements. Effective process control requires knowledge of input, in-process (where appropriate), and output endotoxin levels. Under these circumstances, with appropriate process development, justification for reduced endotoxin challenges or the elimination of endotoxin challenges may be made based on historical data and demonstration of continued control.

CONTROL OF ENDOTOXIN IN PARENTERAL PRODUCTS

The best control of endotoxin levels in parenteral products is the control of Gram-negative bioburden in raw materials, equipment, process streams, and manufacturing environment and operators. Parenteral manufacturers may exercise three categories of control to keep endotoxin content in drug products at safe levels.

The first category is "indirect control", which is comprised of a series of preventive measures that control bioburden, the potential endotoxin contribution by formulation components (e.g., raw materials, APIs, excipients), water, primary packaging components, equipment, and the manufacturing environment, including personnel.

The second category is "process control", in which endotoxin is monitored at CCP during processing to ensure that there is no increase in endotoxin. These process control elements are subject to validation or qualification.

The third category is "direct control", or the direct destruction or removal of endotoxins from product streams, equipment, and primary packaging materials. As with controls on processing, direct measures of endotoxin destruction or removal must be validated.

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[2] ANSI/AAMI ST72:2011. Bacterial endotoxins—Test methods, routine monitoring, and alternatives to batch testing, AAMI, Arlington, VA.
[3] LAL Users Group. Preparation and use of endotoxin indicators for depyrogenation process studies. *J Parenter Sci Technol.* 1989;43(3):109–112.
[4] PDA Technical Report 3 (Revised 2013). Validation of dry heat processes used for depyrogenation and sterilization. Bethesda, MD: Parenteral Drug Association; 2013.

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INDIRECT CONTROL

Reducing opportunities for Gram-negative microbial proliferation at any stage of manufacturing will reduce the likelihood of endotoxin contamination in the following ways:

- Exercising control over the endotoxin content of incoming materials, particularly materials derived from natural sources or those with high water activity, will reduce the opportunity for Gram-negative microbial proliferation and therefore reduce or eliminate the need for endotoxin removal downstream. Because of their manufacturing processes, glass and plastic containers as well as elastomeric closures are often received with very low or undetectable levels of endotoxin. Qualification of primary packaging suppliers should include an audit that examines and confirms the supplier's consistent and documented control over applicable manufacturing processes.
- Bioburden and endotoxin control should be a component of a vendor audit and supplier qualification program for formulation materials that could potentially contribute endotoxin to parenteral products.
- Water is the most ubiquitous raw material in the manufacturing of parenteral products, but unless the generation and distribution of high-quality water is properly validated and controlled, the system will be prone to contamination by Gram-negative bacteria and the establishment of biofilms that can contribute significantly to the endotoxin load of the product (see *Water for Pharmaceutical Purposes* ⟨1231⟩ for a discussion of the types of waters used in pharmaceutical manufacturing and guidance for validation, maintenance, sampling, and testing of systems).

PROCESS CONTROL

Product-specific process control requires the identification of CCP for the introduction or removal of endotoxin. Process control requires good process and equipment design consistent with QbD using a risk management tool such as HACCP. Process control measures include but may not be limited to the following:

- Control of manufacturing practices is essential to endotoxin control. Endotoxin control should be a part of validated cleaning procedures. The use of product contact materials for which endotoxin control cannot be established should be avoided. Clean product-contact equipment should be stored dry to avoid bioburden and Gram-negative bacterial proliferation.
- Hold times during manufacturing, particularly for nonsterile bulk in-process materials and drug product, should be validated to ensure that the hold conditions do not support microbial proliferation and therefore potential endotoxin production by Gram-negative bacteria.
- Environmental control, including good manufacturing practices (aseptic, as appropriate), is essential to process management of endotoxin. Operators should be properly garbed and trained. Housekeeping and disinfecting practices should be established to reduce the possibility of microbial proliferation in critical areas. Cleaning regimens should emphasize that standing water be removed at the end of the cleaning process.
- Endotoxin control and monitoring will be covered in a forthcoming chapter.

DIRECT CONTROL

These processes require validation to ensure that endotoxins are removed or reduced to safe levels. Direct control may be accomplished by a variety of methods and processes that may be combined to ensure endotoxin reduction to a safe level. The most commonly used depyrogenation processes and the associated control measures are the subject of the ⟨1228⟩ series.

SELECTION OF AN APPROPRIATE DEPYROGENATION METHOD

The basic principles for the control and validation of a depyrogenation process using a life cycle approach include:

- Assessment of manufacturing processes to identify materials and process components that are essential to the control of endotoxin
- Focused depyrogenation process development that is consistent with the material to be depyrogenated, the resident endotoxin level of the material to be depyrogenated, and the limit of endotoxin for the finished article
- Adequate validation studies
- Ongoing monitoring of process controls to ensure continued efficacy of the depyrogenation process
- Identifying and documenting changes (a change control program) to the depyrogenation processes over time

Known or anticipated levels of endotoxin can be determined so that appropriate indirect or direct measures of control, consistent with the product or materials of construction, will ensure that endotoxin is eliminated or reduced to levels that ensure product and patient safety.

Should direct methods of depyrogenation be required for the control of endotoxin in or on the article, an important consideration during manufacturing process development is the selection of an appropriate method from the possible alternatives: dry heat, chemical, filtration, or physical removal. In some instances this selection is limited by the potential effects of the depyrogenation treatment may have on the materials themselves. The choice of the appropriate process for a given item requires knowledge of depyrogenation techniques and information concerning effects of the process on the material being processed. The selection of a particular treatment (and the details of its execution) often represents a compromise between those conditions required to destroy the endotoxin or remove it to the desired level and the effect of the process on the materials. Depyrogenation processes should be no more aggressive than required for effective process control to avoid adverse consequences to material quality attributes.

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VALIDATION OF A DEPYROGENATION METHOD

The validation program comprises several formally documented stages to establish that the depyrogenation process is capable of operating within prescribed parameters for process equipment, that independent measurements of critical parameters are possible and accurate, and that acceptance criteria for challenge material removal or destruction are met.

- The development stage investigates and establishes the operating parameters that define the controls to be used for the depyrogenation process.
- The installation qualification (IQ) stage establishes that equipment controls and other instrumentation needed to execute depyrogenation processes and measure the results of the depyrogenation process are properly designed and calibrated. Documentation should be available to demonstrate the acceptability of any required utilities such as steam, water, and air.
- The operational qualification (OQ) stage confirms that the equipment and other processes components function within the defined depyrogenation parameters.
- The performance qualification (PQ) stage of the validation program directly evaluates the depyrogenation of materials or articles. Wherever possible, these studies should employ or simulate the actual conditions of use, including the use of real or simulated product material. Worst case conditions, for example, might include the bracketing of critical parameters such as time/temperature and belt speed for dry heat depyrogenation of glass vials, flow rate for depyrogenation by filtration of solutions, and maximum measured, anticipated, or defined endotoxin loads for any material. "Worst case" should be defined and justified in the validation protocol. Endotoxin indicators may be utilized to support physical measurements in the validation of the depyrogenation process. Although the "rule of three" suggests that three consecutive successful validation runs be executed, perform sufficient replicate studies to demonstrate the capability and efficiency of the depyrogenation process, including the validation of operational ranges of any equipment used in such processes. The number of replicate studies chosen should be scientifically based and justified. At the end of the PQ, a report is written to establish operating parameters.

ROUTINE PROCESS CONTROL

Once a depyrogenation process has been validated it must be maintained in that state to ensure the continued acceptability of its operation. This is accomplished through a number of related practices essential for continued use of the process.

- **Physical measurements**: Data reported by the equipment sensors and recorders must be verified after the completion of each depyrogenation cycle.
- **Calibration**: Any equipment used in the control or quantitative assessment of parameters required for a depyrogenation process must have its measurement accuracy verified against a traceable standard on a periodic basis.
- **Preventive maintenance**: There should be a defined maintenance schedule for each piece of process or testing equipment required for depyrogenation that is consistent with the manufacturer's written recommendation.
- **Ongoing process control verification**: Depending on the specifics of the particular depyrogenation process, there may be additional requirements for ongoing confirmation of process efficacy. These can include the testing of raw materials, water supplies, and in-process sampling. These performance parameters are monitored against assigned limits designed to ensure that finished products meet acceptable endotoxin levels. Monitoring of operating parameters and controls plays an important role in maintaining the depyrogenation process in a validated state.
- **Periodic reassessment**: It is expected that the effectiveness of depyrogenation processes be reconfirmed on a periodic basis. A reassessment schedule should be formalized to assess the potential impact of de minimis or undetected changes to maintain the process in a validated state.
- **Change control**: In order to remain in a validated state, the various material, procedure, and equipment elements impacting the depyrogenation process should be carefully monitored to ensure that changes are properly evaluated for their potential impact on the process. The scope of the change control program must include materials being processed, process equipment, processing parameters, and process holding time limits. The extent of the effort required to support a change will vary with the potential impact of the change on the process outcome.
- **Training**: Depyrogenation processes rely heavily on scientific principles for the effective destruction or removal of endotoxins. Scientists and engineers well-grounded in the principles of endotoxin removal and testing develop processes to ensure effective depyrogenation. Individuals involved in the development of depyrogenation processes require a background in microbiology, physics, chemistry, and engineering, and they must be familiar with good manufacturing principles and regulations. Depyrogenation is an interdisciplinary activity where the combined knowledge of a group of individuals is generally required for the establishment of a reliable process. In addition to the depyrogenation process development team, individuals responsible for the maintenance and operation of depyrogenation processes must also be trained appropriately to ensure that their actions contribute to success. The operators are often the first to identify changes in process performance because of their intimate involvement with it. Effective training programs should be established and documented. Training programs should emphasize depyrogenation principles, adherence to established processes and procedures, and the importance of documenting deviations from normal operations.

ROUTINE TESTING

Testing is not a control mechanism but rather a tool to assess the effectiveness of control measures. Depending on the specifics of the particular depyrogenation process, there may be additional requirements for ongoing confirmation of process efficacy.

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These requirements can include the testing of raw materials, water supplies, and in-process sampling. These performance parameters are monitored against assigned limits based on historical data, and are designed to ensure that finished products meet acceptable endotoxin levels. Monitoring of operating parameters and controls plays an important role in maintaining the depyrogenation process in a validated state.

⟨1228.1⟩ DRY HEAT DEPYROGENATION

INTRODUCTION

Dry heat is the method most frequently used for the depyrogenation of heat stable materials. Dry heat depyrogenation is dependent upon two parameters: time and temperature, which equates to a thermal input. As a result, dry heat depyrogenation processes can be easily monitored/controlled and are highly reproducible. Depyrogenation processes typically operate at a range of temperatures from approximately 170° up to about 400°.

The most prevalent pyrogenic agents in parental manufacturing that are of concern relative to patient safety are bacterial endotoxins, found in the outer cell walls of Gram-negative bacteria. The destruction of bacterial endotoxins (depyrogenation) by dry heat has been studied extensively and has been shown to follow first order kinetics. The well-defined kinetics of inactivation makes it possible to predict the efficacy of dry heat processes operating at different times and temperatures by understanding the total thermal input (F_D).

The range of temperatures used for dry heat depyrogenation overlaps the upper range of temperatures used for dry heat sterilization (see *Dry Heat Sterilization* ⟨1229.8⟩). This is because bacterial endotoxins are more resistant to the effects of dry heat than the most heat-resistant bacterial spores. This chapter provides an overview of the process of dry heat depyrogenation, its control, and validation.

TECHNOLOGIES USED FOR DEPYROGENATION BY DRY HEAT

Although all dry heat depyrogenation processes rely strictly on time of exposure and temperature to assure effectiveness, the equipment used typically falls into two categories: the dry heat "batch" oven and continuous tunnel systems. Batch ovens are routinely used for the depyrogenation of product containers, most often glass, but also other heat stable product contact parts or laboratory equipment. Continuous tunnels, on the other hand, are used primarily to depyrogenate glass product containers.

Batch Ovens

Circulating heated air is used to heat the load items, which may be individually covered or wrapped in a material that is unaffected by the temperature used, or placed in a lidded container for protection during pre- and post-process handling. When depyrogenation and sterilization are to be achieved in the same process, air supplied to the oven is passed through one or more high efficiency particulate air (HEPA) filter(s) to maintain sterility within the oven after completion of the dwell period. These forced air ovens typically operate at a positive air pressure differential relative to the surrounding room. This design results in particulate air quality that can meet ISO 5 requirements to reduce particulate matter and microbial contamination risk throughout processing.

In order to ensure sufficient lethality and process control, oven control probe(s) must maintain a predefined temperature for a predefined time period prior to cooling. The limited heat transfer capacity of air requires that items in the oven be placed in fixed locations confirmed acceptable during the cycle development/validation effort. Caution should be exercised in defining variable load patterns as minimum load sizes may result in inadvertent slower heating of the load and greater temperature variability. Smaller facilities may use a single door oven, but the principles of operation and validation are the same as with larger double door production units. The important batch oven process variables are set-point temperature, duration of dwell period, load type and configuration, airflow characteristics, and container size.

Continuous Tunnels

The use of tunnels for dry heat depyrogenation of glass containers on a moving conveyor allows for substantially higher throughput and packing densities than the batch process, reduces handling, and is ordinarily integrated with a washing and filling system. Tunnels typically use forced heated air systems or radiant IR systems that recirculate air through a battery of HEPA filters. Load items in tunnels are typically fed directly from an integrated container washing system.

Depyrogenation tunnels have separate zones for heating and cooling, allowing for continuous in-feed and discharge at temperatures appropriate for production purposes. The tunnel is maintained at constant airflow and temperature conditions during use, and as glass passes through the tunnel it is heated to depyrogenating temperatures and cooled before exiting. Although the conditions within the tunnel are essentially constant and well controlled, the temperature of the glass as it passes through the tunnel on the conveyor will change with its location. Dwell time is controlled by adjusting the conveyor speed,[1] which in the depyrogenation tunnel is the process parameter that governs exposure time.

The air in the tunnel is most commonly heated using electrical coils but other heat sources, such as infrared or high-pressure steam, have been used. For energy conservation, heated air in depyrogenation tunnels is often recirculated. The important

[1] Not all tunnels have a variable speed capacity.

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continuous tunnel process variables are heating zone temperature, cooling zone temperature, belt speed, and container mass per unit.

DRY HEAT DEPYROGENATION FUNDAMENTALS

Dry heat depyrogenation uses air first to heat and then to cool the items. The limited heat capacity of dry air results in relatively slow heating and cooling of the load items. Variability in temperature distribution in dry heat ovens and tunnels is typically much higher than that observed in moist heat systems. The limited heat capacity of air requires that items in ovens be placed in the same locations as confirmed acceptable in the cycle development/validation effort. Packing and thermal mass will also play critical roles in temperature management. Caution must also be exercised with varying load mass and distribution as in some instances (resulting from oven design, air flow characteristics, and control probe position) minimum load sizes may result in process variability.

DEPYROGENATION PROCESS CONTROL

Process temperatures in dry heat depyrogenation are controlled by calibrated temperature sensors placed at specific locations within the equipment. The exposure portion of the process is designed to attain a minimum dwell time at a predefined minimum temperature ensuring that depyrogenation conditions are adequately uniform. The defined dwell time is determined by using measurement devices (e.g., thermocouples) directly in contact with the items to be depyrogenated during development. The inactivation of bacterial endotoxins by dry heat involves the control of only two parameters: time and temperature.

The simplicity of process control for these parameters provides a predictable depyrogenation effect. Once validated, a clear understanding of the inputs to the system, meaning resident endotoxin load on incoming materials, and outputs of the system, meaning reduction of resident levels of endotoxin to safe levels, is more meaningful than spiking with unnaturally high levels of challenge material and looking for a prescriptive requirement for log reduction. The dosimetric measurement for dry heat depyrogenation processes is the F_D unit. An $F_D = 1$ is defined as the depyrogenation effect achieved by 1 min of heating at 250°. The F_D-value enables the integration of temperature over the process duration (time). By convention, the rate at which depyrogenation destruction rate (*D*-value) varies as a function of temperature change is defined as the *z*-value. The *z*-value for dry heat depyrogenation has been shown to be in the range of 45°–55°. For the purposes of this chapter, 50° is used as a standard *z*-value. Other values may be used. (*1*,*2*)

The F_D approach is used as a means to compare dry heat depyrogenation effects produced by processes that operate at varying temperatures. Basic mathematics can be used to calculate the depyrogenation effect produced at temperatures other than 250° to determine equivalence to that provided at 250°.

Using a reference temperature of 250° and an assumed *z*-value of 50°, the F_D calculation can be determined:

$$F_D = \int_{t_1}^{t_2} 10^{\left(\frac{T-250}{50}\right)} dt = \sum_{t_1}^{t_2} 10^{\left(\frac{T-250}{50}\right)} \Delta t$$

F_D = accumulated destruction
t_1 = process start time
t_2 = process end time
T = temperature at each time increment
Δt = time interval between temperature measurements

Summing the instantaneous temperature contributions over the entire depyrogenation process allows for the calculation of the overall process efficacy or F_D delivered over the course of the process. Many commercial data loggers are equipped with software that enables them to make this calculation and integrate the total F_D accumulated during a process. The F_D calculation is used during initial validation, validation maintenance, and change control. The mathematical principles of the F_D calculation are essentially the same as those used to calculate lethality (F_0) values in moist heat sterilization. F_D values are used to confirm process consistency over time as correlation to endotoxin destruction is rarely possible.

VALIDATION

Because dry heat depyrogenation is appropriate only for heat stable materials, a high margin for safety is always attainable. Times and temperatures used for the purpose of destroying challenge materials can result in extreme challenges to material integrity and stability. Attention to depyrogenation processes, including an understanding of the resident endotoxin load on incoming materials and reduction to levels needed to assure patient safety, should take place during drug product development, prior to validation.

Equipment Qualification

Equipment Qualification (EQ) is a predefined program that focuses on the processing equipment to confirm that it has been properly installed and operates as intended prior to evaluation of the process. In some companies, EQ may be separated into installation qualification (IQ) and operational qualification (OQ), or combined together under a joint terminology of installation/operational qualification (I/OQ). Equipment qualification provides a baseline for preventive maintenance and change control assuring reproducibility of equipment operation over time.

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Empty Chamber Temperature Distribution for Ovens

The oven should be evaluated for empty chamber temperature distribution. This is assessed by measurement of temperature at each corner of oven, near the controlling probe(s) and other locations as justified. Differences in the cycle dwell period can be discounted in this evaluation, as only the shortest dwell period need be evaluated. The evaluation is best performed over the last few minutes of the dwell period once the system has fully stabilized. The acceptance criteria for this test vary with the oven's design and operating conditions; however, temperature distribution is typically substantially less uniform than observed in autoclaves and may be ±15° or more. Depyrogenation ovens that are located at floor level may have even greater ranges in temperature. The temperature distribution measurement may be of value in the evaluation of changes to the oven.

Empty Temperature Distribution in Tunnels

While these studies are often done, they are actually of limited value. Unloaded depyrogenation tunnels will always produce far more variability in temperature distribution than will a fully loaded tunnel. Therefore, for depyrogenation tunnels, temperature studies under fully loaded conditions only are indicated. Important to the proper operation of the dry heat tunnel is the establishment of the required air flow balance between the tunnel and the adjoining areas. Improper air flow can cause uneven heating across the load being processed. The temperature distribution measurement may be of value in the evaluation of changes to the tunnel.

Component Mapping

The ability of dry heat to penetrate load items and to bring them to the required temperature should be determined. Load items that are complex, of significant mass, with enclosed volumes and product contact surfaces that must be depyrogenated, should be subjected to component mapping to determine internal cold spots. All load items should be prepared, wrapped (if that is the practice), and oriented in a manner consistent with how they will be processed. Glass typically enters the depyrogenation process (whether tunnel or oven) wet and must be evaluated wet to properly determine the effect on overall thermal input. Mapping of glass components to be processed in tunnels is not necessary; all monitoring of temperature in tunnels is accomplished with probes in contact with the bottom of the container.

Load Mapping in Ovens

Fixed loading patterns are necessary in oven depyrogenation because of the limited heat capacity of the air; fully packed conditions because of their greater mass ordinarily result in the best process temperature uniformity. Load mapping assures that items placed throughout the load attain the desired depyrogenation conditions. Identification of cold zones within the oven should be established during depyrogenation cycle development. Information from the load mapping is used to adjust cycle timing to assure appropriate efficacy across the entire load. It may be possible to validate maximum and minimum loads (as determined by either the number of items or their mass).

F_D is calculated from the temperature data at all monitored locations within the load pattern.

Load Mapping in Tunnels

Load mapping can be assessed using sets of calibrated sensors (i.e., trailing or wireless temperature sensors) positioned within the glass pack as it moves through the tunnel. Temperature sensors should be placed into direct contact with the glass item at the bottom of the container. Temperature measurements should be made on the leading edge, the middle (highest density), and the trailing edge of the glass pack across the width of the conveyor belt. There should be NLT 5 temperature sensors positioned across the belt in each section of the load. F_D is calculated from the temperature data at all monitored locations. Studies should be performed using all container sizes to determine the lowest F_D locations. The F_D results can be used to support the selection of containers/conditions to be evaluated in the confirmation studies (see below). There is no requirement to perform temperature heat distribution measurements during these studies.

Confirmation of Depyrogenation

The materials/glass components to be depyrogenated should be assessed for their incoming endotoxin content prior to the validation study. This would include glass as received and immediately after washing. All tested materials should be handled and prepared using defined procedures. Materials and glass prepared in the same manner are used in the depyrogenation validation studies. The addition of challenge material to the load items, including a requirement to demonstrate a 3-log reduction, may not be required if time and temperature studies consistently indicate that depyrogenation conditions are met. Temperature monitoring as described above must be done simultaneously with the depyrogenation confirmation studies. The confirmation studies should be performed at reduced time-temperature conditions from those utilized in routine processing and deliver lower F_D results when compared to those determined in the mapping studies and are considered "worst case" confirmation of depyrogenation process efficacy. There is no requirement to perform temperature heat distribution measurements during these studies.

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OVENS

A minimum of five (5) samples should be taken in proximity at NLT 10 temperature-monitored locations (including those determined to be the coldest from the oven load mapping study) in the oven and tested for endotoxin content post-processing. The process is considered acceptable if the amount of endotoxin per sample is NMT 0.1 EU.

TUNNELS

A minimum of five (5) samples should be taken in proximity to each monitored position within the tunnel load (including those determined to be the coldest from the tunnel load mapping study) and tested for endotoxin content post-processing. The process is considered acceptable if the amount of endotoxin per sample is NMT 0.1 EU.

ROUTINE PROCESS CONTROL

As with all processes, after the dry heat depyrogenation process has been validated, it must be subject to ongoing controls that maintain it within the validated state at all times. Temperature and exposure time, which are the important dry heat depyrogenation parameters, can be used to confirm performance on a routine basis. Where direct assessment of F_D is not possible, assuring that the temperature and exposure time conditions were met results in an equivalent confidence that the depyrogenation system operated in a validated state of control.

Depyrogenation ⟨1228⟩ details the general practices that are appropriate for all depyrogenation systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, periodic endotoxin assessment on incoming materials, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

APPENDIX

Additional Sources of Information

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⟨1228.3⟩ DEPYROGENATION BY FILTRATION

1. INTRODUCTION
2. TECHNOLOGIES USED FOR DEPYROGENATION BY FILTRATION
    2.1 Microporous Membrane Filtration
    2.2 Reverse Osmosis
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    2.5 Activated Carbon Depth Filters
    2.6 Membrane Adsorbers
3. VALIDATION

1. INTRODUCTION

Depyrogenation by filtration involves the physical removal of endotoxins from pharmaceutical solutions by adsorption and size exclusion. Endotoxins are responsible for making up the majority of pyrogens, which must be removed from pharmaceutical products including injectable biologics. There are many factors to be considered when designing a depyrogenation filtration process for solutions containing proteins and peptides: type of target protein and its concentration, electrolyte concentration, pH and buffer system, protein molecular weight and isoelectric point (pI), filtration parameters (e.g., flow rate), and interactions

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with other components causing aggregation. In general, a combination of these factors determines the most effective depyrogenation method.

Depyrogenation of liquids may be accomplished by means of filtration through various types of filter media including microporous membranes, reverse osmosis (RO) membranes, ultrafilters, charge-modified depth filters, activated carbon, and membrane absorbers. Depyrogenation filtration processes are not intended to remove microorganisms from a process stream; however, by their nature, filters selected for use in depyrogenation processes may also be capable of retaining many types of microorganisms.

2. TECHNOLOGIES USED FOR DEPYROGENATION BY FILTRATION

2.1 Microporous Membrane Filtration

Microporous membranes (typically with pore size or retention ratings between 1.0 and 0.1 µm) can be very effective in removing intact bacteria via size exclusion and adsorption within flow pathways. The use of microporous membranes on a freshly prepared solution to be filtered can effectively prevent bacterial proliferation in the solution, along with any potential subsequent endotoxin formation. Endotoxin, however, is composed of fragments of bacterial cell wall, often <0.025 µm (*1*) that may easily penetrate most bacteria-retentive membrane filters. These negatively charged particles with endotoxin activity can be removed via adsorption by positively charged membranes (*2*). Adsorption of endotoxin has also been shown by hydrophobic membranes, where it is thought that a hydrophobic interaction occurs between the Lipid A core and hydrophobic sites on the membrane flow path surfaces (*3*). Reduction or removal of endotoxin activity by adsorption to microporous membranes can be dependent on flow rate, pH, concentration, and fluid and membrane surface properties. Once the effective binding capacity of the membrane approaches saturation under applied conditions, remaining endotoxin will pass through the membrane.

2.2 Reverse Osmosis

RO membranes are the tightest membranes in size separation. They can separate dissolved salts and sugars from water. Pyrogens, and essentially everything else, are removed from water via size exclusion. RO systems are operated most efficiently at high pressure (200–1000 psi) to overcome osmotic pressure. RO membrane rating or tightness is measured and expressed with retention or rejection of marker salts such as sodium chloride or magnesium sulfate.

RO membranes may be composites (thin film coated on top of ultrafiltration membranes) or cast as a single layer (cellulose acetate type). Configuration of RO membrane modules can be flat sheet, tubular, or hollow fiber. All commercially available RO membranes are polymeric, and most are of a spiral-wound, flat-sheet format.

RO systems are not intended to remove all bacteria, and because they are run at ambient temperatures, microbiological contamination is a concern. Ultraviolet (UV) light may be used in the system downstream from the RO units to control microbiological contamination.

2.3 Ultrafiltration

Ultrafiltration (UF) is a process whereby a fluid is passed through membranes with pore sizes nominally between about 1 and 100 nm under pressure. The filters are usually not rated by the pore size but by the molecular weight cut-off (MWCO). The methods to determine the MWCO vary by the manufacturer and usually involve measuring passage of molecules of a certain size, such as a solution of mixed dextrans, polyethylene glycol, or proteins to assign a numerical rating (*4*).

UF membranes are usually polymeric porous structures, manufactured from a range of materials, most commonly regenerated cellulose or polyether sulfone, but also ceramics. UF membranes may be produced as flat sheet, hollow fibers, or ceramic tubes.

UF is generally operated in tangential/cross flow mode, which separates the starting (feed) solution into two components: permeate (the portion of solution going through the membrane) and retentate (the concentrated solution that is passed over the membrane). UF membranes need to be encased in a suitable integral device to enable practical operation. Heat sealing, over-molding, and resin-potting are all used to assemble membrane devices and ensure integral flow paths. Ceramic tubes are sealed by gaskets within tubular cylinders.

It is generally assumed that the basic subunit of lipopolysaccharide (LPS) is about 10–20 kDa (*5*). Membranes of 6–10 kDa cut-off are often used for depyrogenation by size exclusion. However, monomeric LPSs are rarely found in solution because of their poor solubility in water. LPS is usually present in aggregated forms, such as vesicles ranging in molecular weight from 300 to 1000 kDa. Thus, endotoxin can be successfully removed by higher flux membranes, with MWCOs of 30–100 kDa (*6*).

Adsorption, in addition to size exclusion, also can be a mechanism of endotoxin removal by UF. Several hollow-fiber membrane materials have been evaluated, and the best removal was obtained with more hydrophobic membranes. Endotoxin removal was correlated to the degree of endotoxin adsorption on the membranes in an equilibrium experiment (*7*).

UF has been used successfully to depyrogenate small molecule drugs, buffers, electrolytes, antibiotics, and antifungal agents (*8*). UF is generally not recommended for endotoxin removal from solutions containing larger molecules such as proteins.

2.4 Charge-Modified Depth Filters

Depth filters exhibit two primary clarification mechanisms because of their structural and chemical composition: size exclusion, either through sieving or entrapment; and adsorption, either through electrokinetic (positive zeta potential) or hydrophobic interactions.

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Size exclusion of particles is a function of the tortuous flow path through the media as well as the depth or length of the flow path in relation to the size distribution of the contaminate loading, e.g., cellular debris, including LPS from cell walls and hard particles. Depth filtration efficiency depends on many factors, including the filter media characteristics, materials of construction (e.g., cellulose, filter aids, binding resins), the fluid characteristics (e.g., viscosity, dirt load, cell debris, temperature), as well as the particle characteristics (e.g., solid/hard, pleomorphic, proteinaceous, colloidal). Electrokinetic adsorption is attributed to the resin binders and filter aids that impart a net-positive charge, positive zeta potential, to the filter medium. Adsorption is a complex mechanism that will vary based on a combination of parameters including positive zeta potential, hydrophobic adsorption, particle surface charge, pH, and ionic strength of process fluids. This positive zeta potential can remove negatively charged particles smaller than the nominal rating of the depth filter medium. The adsorptive mechanism results in high removal efficiencies for fine particles, colloidal and cellular materials, e.g., bacterial endotoxins, nucleic acids, and removal of negatively charged trace contaminants, whereas the depth medium porosity influences operating parameters such as pressure differentials, flow rates, dirt load capacity, and throughput.

Most cellulose-based depth filters contain a filter aid to enhance particle retention and flow characteristics. Filter aids are available in various particle sizes and levels of purity. Common filter aids include diatomaceous earth, perlite (volcanic origin), carbon (natural sources), and silica- and/or metallic-based materials.

The cartridges and capsule configurations are constructed of primarily polypropylene and other common elastomers and polymers, e.g., nylon, polycarbonate, polysulfone. Depth filters are available in standard filter cartridge/capsule configurations; lab-scale discs (47 mm/90 mm), flat stock sheets, lenticular cartridges (stacked discs), and capsules.

In general, the charge-modified depth filters showed lower endotoxin breakthrough levels at charge exhaustion as compared to charge-modified membrane filters.

Membranes demonstrated total endotoxin breakthrough once the charge capacity of the membrane is saturated. Generally, charge-modified depth filter media demonstrate lower endotoxin unit (EU) levels that increase slowly at the point of first endotoxin detection as compared to membranes that exhibit complete breakthrough.

Benefits of charge-modified depth filters include removal of bacterial endotoxin [4–5 log reduction value (LRV)] (*9–11*), DNA fragments, host cell protein, reduction of viruses, and economical throughput with low extractable levels. System flow rate determinations are necessary to optimize residence time to maximize adsorptive capture. This parameter is especially important for the removal of colloids and endotoxins.

Cellulosic depth filters commonly contain extractable *Limulus* amebocyte lysate (LAL)-reactive materials that are often determined to be β-1,3-glucans. β-1,3-Glucans activate an alternative LAL pathway, Factor G. The activation of Factor G by β-1,3-glucans will induce the proclotting enzyme, causing a non-endotoxin-positive LAL result (or enhanced result). To reduce the risk of β-1,3-glucan extractables from cellulosic depth filters, it is important to follow the recommended rinse conditions of the specific depth filter. An alternative to reduce the effects of β-1,3-glucans is to select LAL reagents tolerant of β-glucans or to add a β-glucan blocking buffer to LAL samples.

To some users, the most important attribute of charge-modified depth filters is their effectiveness as prefilters. In more difficult filtrations, such as those containing colloids, bacteria, or endotoxins, the user can realize substantial cost savings.

2.5 Activated Carbon Depth Filters

Depth filtration, using activated carbon as a filter aid adsorbent, removes color, odor, and bacterial endotoxins and nucleic acids. Activated carbon is derived from organic materials, e.g., peat, wood, coconut, bone, lignite coal. The microstructure of the carbon contains millions of pores that create a highly adsorptive material with a vast internal effective surface area as compared to polymeric microporous structures. These carbon filter aids are typically activated by steam or chemical treatment such as acid. Although highly effective in reducing endotoxin (4–5 log reduction) and other undesirable contaminants, active carbon may, because of this highly adsorptive characteristic, remove other process components and target molecules due to this nonspecific adsorption property. The high loading capacity and strong adsorptive attributes make activated carbon depth filtration an attractive alternative to conventional filtration methods or addition of bulk carbon, where care must be taken to remove fine carbon particulates in the effluent.

2.6 Membrane Adsorbers

When the target protein in the solution is in the same molecular weight range as that of the endotoxins (10–20 kDa for endotoxin monomers), the target proteins cannot be separated by UF. Ion exchange chromatography is the most common depyrogenation method for proteins; however, it has some drawbacks, which limit its usefulness as a depyrogenation step. This includes handling and usage problems such as packing, channeling, low flow rates, long regeneration times, compressibility, and limited chemical stability. Charge-modified membrane adsorbers with ion exchange ligands functionalized on the membrane surface can provide the required performance needed for depyrogenation from the laboratory up to process scale. Generally, two strategies can be used for removal of endotoxin from solutions with such membrane adsorber devices. Using the strong basic anion exchanger of quaternary amine (Q) type in a buffer with pH lower than the pI of the protein, endotoxin will bind to the charged membrane substrate, and protein will pass through the membrane (negative chromatography). Alternatively, a strong acidic ion exchanger type S also can be used with a buffer pH lower than the pI of the protein. In this case, the endotoxin will pass through, and the protein will be bound to the charged membrane substrate, which can be subsequently eluted using appropriate buffers in the next step.

Such membrane adsorbers have been used as validated endotoxin clearance steps in downstream processing of monoclonal antibody (mAb) or recombinant protein manufacturing. Typical log reduction values (LRVs) reported are >4 (*12*) based on lab scale testing.

Another solution is to use mixed-mode membrane adsorbers exhibiting both anionic and hydrophobic chemistries. Endotoxins (hydrophobic and negatively charged) tightly bind onto the membrane surfaces. By adjusting the concentration of salt or pH appropriately, proteins flow through the mixed-mode membrane adsorber by charge repulsion, while endotoxins remain bound. Mixed-mode membrane adsorbers allow the depyrogenation of protein solution or buffers with higher

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concentrations of salt (e.g., 100–500 mM) than with the Q adsorber. Such membrane adsorbers have been used as validated endotoxin clearance steps in downstream processing of mAb or recombinant protein manufacturing. Typical LRVs reported are in the 3–4 range based on lab scale testing.

3. VALIDATION

See *Depyrogenation* ⟨1228⟩ for a comprehensive discussion of depyrogenation process validation and the use of endotoxin standards.

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APPENDIX

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Add the following:

▲⟨1228.4⟩ DEPYROGENATION BY RINSING

INTRODUCTION

The production of parenteral products requires that products be sterile, but also that they are free from harmful levels of pyrogens, or fever causing agents. For the purposes of the ⟨1228⟩ series, the term "depyrogenation" refers to the destruction or removal of bacterial endotoxins, the most prevalent and quantifiable pyrogen in parenteral preparations. Effective depyrogenation depends on the product and the method of removal and/or destruction. Although depyrogenation of heat-stable articles may be best accomplished by dry heat, heat-labile equipment, components, ingredients, or materials such as drug product containers and closures, and some medical devices may be depyrogenated through physical means such as rinsing. This chapter provides an overview of the depyrogenation process, its validation, and routine process control.

RINSING PROCESS

Rinsing is the most common means of reduction or removal of bacterial endotoxins on closures (such as elastomeric stoppers), medical devices, and other materials that are not compatible with the temperatures used in dry heat depyrogenation. The

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mechanism for this method of depyrogenation is removal of the endotoxin, followed by dilution (*1*). The process of general rinsing to remove pyrogens is accomplished by using high-purity water such as *Water for Injection* preferably above 60°. Multiple rinses may be necessary with proper controls to ensure that the *Water for Injection* does not become contaminated with Gram-negative bacteria and bacterial endotoxins during processing.

The use of high-purity rinse water is often the key to successful reduction of bacterial endotoxin activity on the surfaces of the materials being processed. At a minimum, the rinse water quality should meet the bacterial endotoxin limit of *Water for Injection*, which is <0.25 USP Endotoxin Units (EU)/mL. Using water that risks having bacteria grow in it may result in the deposition of bacterial endotoxins onto the items, or in the case of depyrogenated articles, the re-deposition (re-pyrogenation) of endotoxins from the rinse water itself. The advent of *Water for Injection* for use in the healthcare manufacturing industry has ensured that the most prevalent ingredient in most of our products is no longer a source of endotoxin. Additionally, re-pyrogenation of articles due to subsequent microbial proliferation on the rinsed items should be minimized either by a prompt subsequent sterilization or by drying the items after rinsing.

It is important to note that starting with *Water for Injection*, while essential, does not ensure that such water remains suitable for use through the duration of the depyrogenation process. When cooled from the elevated circulation temperatures used for its distribution, *Water for Injection* is vulnerable to microbial proliferation. *Water for Injection* held at temperatures below 55° and above 8° should be considered at risk for microbial contamination unless sterilized and held in a sterile vessel. Therefore, holding water for more than 3–4 h within this temperature danger zone is an unsuitable practice. Systems that recirculate *Water for Injection* can be effective but process conditions and water storage times and temperatures should be carefully controlled and validated.

Solvents other than *Water for Injection*, such as caustic alkali or detergents, have been used for depyrogenation by rinsing. The concern with these solvents is the removal of residuals that may ultimately be harmful to patients or the product, so care must be taken to test for residuals during validation of the method.

For any process that purports to depyrogenate by rinsing, there are a number of critical factors that must be defined and controlled during the validation study and beyond:

1. Solvent description, including normality or concentration if caustic alkali or detergents are used; if high-purity water is used, the source should be clearly described
2. Solvent temperature
3. Solvent pressure, particularly where rinsing is used to depyrogenate glass or other articles that remain stationary during the process
4. Solvent flow rate through the system
5. A justification for the recirculation of solvents

VALIDATION

Validation of depyrogenation by physical means is not different, in principle, from the validation of other depyrogenation methods. Endotoxin indicators, which are articles representative of the material to be depyrogenated spiked with a known amount of endotoxin, are prepared in a laboratory (see *Endotoxin Indicators for Depyrogenation* ⟨1228.5⟩). Quantitation of endotoxin activity prior to and subsequent to the rinsing process will demonstrate the effectiveness of the process in removing endotoxins. To prepare endotoxin indicators, inoculate each indicator with a known level of activity of Control Standard Endotoxin (CSE), USP Endotoxin RS (which, by convention, is abbreviated as RSE), or Naturally Occurring Endotoxin (NOE), which is calibrated against RSE before processing. Generally speaking, a small volume (e.g., <100 µL) of a highly concentrated analyte (purified LPS or NOE) is inoculated onto a section of the article that is the most difficult for the rinse solvent to reach. For example, stoppers are generally inoculated onto the product contact portion of the stopper. A low volume of inoculum is used to ensure rapid drying. The inoculated indicators may be dried in a unidirectional air flow cabinet or other validated means to hasten drying and limit the possibility of extrinsic microbial contamination that can arise in uncontrolled environmental conditions, particularly with moisture present. Historically, 1000 EU/article has been the target spike value, but depending on 1) the level of endotoxins historically found on the material, 2) the amount of recoverable endotoxins observed after the drying process, and 3) the "safe" level of endotoxin activity that must be attained post-processing, an inoculum at a level other than 1000 EU may be justified.

Because the prepared endotoxin indicators may be mixed with many uninoculated articles (e.g., stoppers), it is essential to provide a means to identify the indicators. For example, if the stoppers used by the company are gray, an identical stopper that is a different color but has the same elastomeric material, or a stopper of the same composition but a different shape, could be used for the indicators. If a stopper of different color or other identifying mark is not available, the inoculated indicator stoppers might be placed in individual small mesh bags. For glass containers, the indicator containers might be amber rather than clear, or they may be marked with indelible or heat resistant ink. For metal articles, a heat-resistant marker might be used to identify the indicators. In any event, the endotoxin indicators must be identical in materials of composition to the other articles in the study, but must be prepared so that they can be easily retrieved for analysis.

Once dry, the endotoxin indicators are analyzed for the recoverable level of endotoxins activity using the *Limulus* amoebocyte lysate (LAL) assay. To determine the recoverable activity, follow the process for extracting endotoxin from medical devices (see *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩). Submerge the article or a number of articles in Water for Bacterial Endotoxins Test (BET; see *Bacterial Endotoxins Test* ⟨85⟩, *Reagents and Test Solutions, Water for Bacterial Endotoxins Test*) prewarmed to 37°. Allow the article(s) to remain in contact with the Water for BET for an hour. Agitation such as intermittent vortexing or sonication may be added to enhance recovery. If methods other than immersion in Water for BET are used, they must be validated to demonstrate that they do not result in a loss of endotoxin activity and identified in the protocol so that subsequent studies use the same process. Regardless of the method used, a statistically significant number of samples representing the load under study should be evaluated to ensure adequate reproducibility.

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Controls

Two sets of controls are recommended for validation studies:

1. Testing of uninoculated indicators—provide data on the resident level of endotoxin activity on the lot of stoppers to be tested.
2. An appropriate number of endotoxin indicators should be retained by the laboratory and used as positive controls. After the depyrogenation process is completed, extract both the processed units and positive control indicators as described above and test using a qualified BET method.

Any activity detected on the retained positive controls is called "recoverable" activity and activity detected on the processed indicators is called "residual" activity (*2*). The following factors should be considered during the extraction of endotoxin activity:

1. Inoculated units should not be pooled for analysis. The conceptual basis for this study design is to determine the depyrogenation capability of the process, and pooling extracts from a number of units would not allow evaluation of reproducibility and also bias the results. However, there may be a unique circumstance in which accurate or valid results may not be possible because of a small sample size or other logistical or practical considerations. If articles are pooled, the reason for pooling should be documented in the body of the protocol.
2. The test result must account for all sources of dilution. For example, the units for an LAL test are EU/mL. If the extraction volume is 1 mL/stopper, then the result of the test is really EU/stopper. But, for example, if the extraction volume is 5 mL/stopper, then each milliliter of the extract is a 1:5 dilution of any endotoxin activity that has been extracted from the indicator.

The log reduction is calculated using the following formula:

$$\text{Log}_{10}\text{ reduction} = (\log_{10}\text{ recoverable activity}) - (\log_{10}\text{ residual activity})$$

For example, if a laboratory detects 4500 recoverable EU/stopper in the controls and 0.3 residual EU/stopper, the log reduction is calculated as:

$$\text{Log}_{10}\text{ reduction} = (\log_{10} 4500\text{ EU}) - (\log_{10} 0.3\text{ EU})$$

$$\text{Log}_{10}\text{ reduction} = (3.65) - (-0.52) = 4.17$$

Historically, laboratories added sufficient inoculum to each endotoxin indicator unit so that at least 1000 EU could be recovered in the retained controls. However, there are factors that the analyst and the validation team should be aware of in conducting these studies. Foremost among these factors is an understanding that the lipopolysaccharides extracted via the Westphal procedure, of which CSE and RSE consist, have physicochemical properties that can make achieving a uniform dispersion of a highly concentrated quantity of this material difficult or impossible. Also, CSE tends to adsorb to surfaces and therefore may be difficult to remove from some materials resulting in control recoveries of less than 50%. In some cases, the recovery may be substantially less than 50%. There may also be considerable variability in recovery from test to test or even within a single test run. This is to be expected and history has taught us that this does not impact the usefulness or effectiveness of a depyrogenation study.

A 3-log reduction of the recoverable endotoxin is a common target for acceptable depyrogenation effectiveness (*3*). However, this may not be attainable in all test systems or under all test conditions nor may it be necessary. Where a 3-log reduction is not attainable it is important to carefully evaluate endotoxin risk in the process based extensively on inherent endotoxin burden. Rather than strict reference to a 3-log reduction, the depyrogenation program must prove that endotoxin activity at levels below a scientifically established target can be consistently attained. The target should consider the use of the article and the potential contribution to the final endotoxin limit established for the drug product. Although the endotoxin limit is established based on calculations of active pharmaceutical ingredient (API) dosage contained in the drug product, any contributions by the excipients and container/closure must be considered in any validation study, and the sum of all these contributions must meet this limit.

Elastomeric closures are generally received with little or no detectable endotoxin activity. It is possible that a 2-log reduction by rinsing could be sufficient for some packaging components, excipients, or other materials. An important consideration in making this determination is the incoming pyroburden and bioburden presented by any material to the depyrogenation process.

ROUTINE PROCESS CONTROL

Proactive control of Gram-negative bacterial contamination is essential to the production of safe parenteral products. The use of properly generated, stored, and distributed *WFI* ensures that the most prevalent ingredient in most products is not a significant source of endotoxin.

Once a depyrogenation process has been effectively developed and validated, it must be maintained in that state to ensure continued acceptability. Chapter ⟨1228⟩ details the general practices that are appropriate for all depyrogenation systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, periodic endotoxin assessment on incoming materials, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

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⟨1228.5⟩ ENDOTOXIN INDICATORS FOR DEPYROGENATION

1. INTRODUCTION
2. ENDOTOXIN AND LPS
3. APPLICATION OF ENDOTOXIN INDICATORS
4. PREPARATION AND USE OF ENDOTOXIN INDICATORS
    4.1 Methodology to Create a Laboratory-Prepared Endotoxin: Principles to Consider
    4.2 Inoculation of EIs
    4.3 Recovery of Endotoxin from EIs
    4.4 Choice of Test Methodology for the Analysis of EIs
5. ANALYSIS OF RESULTS OF DEPYROGENATION STUDIES

1. INTRODUCTION

Depyrogenation is defined as destruction or removal of pyrogens (see *Depyrogenation* ⟨1228⟩). For the purposes of this chapter, the terms "bacterial endotoxin" or "endotoxin" refer to a component of the outer cell membrane of Gram-negative bacteria, which is known to induce a febrile response in humans and other mammals. The endotoxin complex contains many cell wall components including, but not limited to, phospholipids, lipoproteins, and lipopolysaccharides. Lipopolysaccharide (LPS) is the biologically active portion of both naturally occurring and laboratory-prepared endotoxin complexes. The USP Endotoxin Reference Standard (which, by convention, is abbreviated as RSE) and control standard endotoxin (CSE) preparations purchased from lysate manufacturers and other third-party vendors are not endotoxins but rather are preparations of purified LPS.

"Endotoxin indicators" (EIs) are tools that are used (where required) in conjunction with physical measurements to analyze the effectiveness of a depyrogenation process. EIs used to determine the effectiveness of dry heat depyrogenation processes are commonly purchased as glass vials that are inoculated with a known level of LPS activity. This chapter expands the definition of EI to include any carrier, including glass vials, inoculated with endotoxin or LPS that is used to challenge a depyrogenation process. EIs can be used to analyze the effectiveness of endotoxin removal by washing, rinsing, cleaning, or by using separation technologies, such as filtration or chromatography. Carriers can be a variety of materials, including rubber stoppers to assess stopper-washing processes, bulk product to assess and validate processing steps, and stainless steel coupons to assess the cleaning of production vessels.

Purified LPS, such as CSE obtained from lysate manufacturers or other third-party vendors, has historically been a convenient choice for use as the analyte used in the preparation of EIs. However, EIs prepared in-house using laboratory-derived endotoxin more closely mimic product contamination, and as a result can provide a more realistic assessment of the depyrogenating capability of various production processes than does highly purified LPS. This chapter provides information on the preparation and use of these more specialized indicators to assure both consistency and comparability of data among method development and validation studies.

2. ENDOTOXIN AND LPS

A bacterial endotoxin is defined in the *Introduction*. LPS is the biologically active portion of the naturally occurring and laboratory-prepared endotoxin complex. Highly purified LPS, extracted from the natural endotoxin complex, is used to prepare the primary compendial RSE or secondary CSE preparations, such as those purchased from *Limulus* amebocyte lysate (LAL) reagent manufacturers. LPS consists of three distinct regions:

1. The structure of the hydrophobic lipid A portion of the molecule is the most highly conserved among Gram-negative species and is responsible for most, if not all, of the biological activity of endotoxins
2. A core oligosaccharide links the lipid A to the hydrophilic *O*-specific side chain or *O*-antigen
3. The hydrophilic *O*-antigen is a highly variable region that confers serological specificity to the organism and is often used to distinguish strains of Gram-negative bacteria

When drug products and devices are contaminated with endotoxin, the contaminant is not purified LPS but rather whole Gram-negative cells and/or cell wall fragments containing LPS. LPS and endotoxin are therefore dissimilar in many respects.

The amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a nonpolar (hydrophobic) end] enables it to form complicated, three-dimensional, aggregated structures in solution. The aggregated forms of LPS have the capacity to adsorb, or "stick", to surfaces, and depending on the LPS formulation and the surface, extraction and detection

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may prove difficult using conventional extraction methods (see below). The degree of aggregation of the purified molecule is also affected by the conditions to which the LPS is exposed. Factors such as temperature, pH, salt concentration, divalent cation concentration, chelating agents, and detergents can have a profound effect on the biological activity and stability of LPS in solution. Purified LPS preparations used for depyrogenation studies should not contain any "fillers" or excipients. The excipients that are commonly used in the formulation of CSE have been shown to reduce the heat resistance of LPS (*1*) and may interfere in the recovery of LPS because of a caramelized excipient that has been post-processed by dry heat.

Endotoxins contaminating parenteral products may exhibit greater stability of activity in solution and less surface adsorption than purified LPS. As well, the detection of endotoxin may be less influenced than LPS by aggregation, disaggregation, or other conformations induced by some product matrices. Information on principles to consider when preparing endotoxin in the laboratory can be found below.

3. APPLICATION OF ENDOTOXIN INDICATORS

The choice of an EI should be relevant to the process being validated. For physical depyrogenation, such as dry heat, the carrier material for the EI may be a surface such as a glass vial or appropriate coupon material with documented heating characteristics similar to the materials being processed, and onto which a known quantity of LPS or endotoxin has been inoculated. For stopper washing/depyrogenation studies, stopper carriers are inoculated with known levels of LPS or endotoxin. For raw materials or process intermediates that are inherently contaminated with assayable levels of endotoxin activity, there may not be a need to add LPS or endotoxin to validate endotoxin reduction in the manufacturing process, as the level of contamination may be sufficient to accurately measure activity upstream and downstream of the depyrogenating step(s).

For processes using raw materials or for upstream intermediates that are not contaminated with endotoxins, the use of either the USP RSE or CSE, which are both highly purified preparations, may not reflect the actual removal or reduction potential of the product stream depyrogenation step(s) under challenge. For these purposes, endotoxins harvested from Gram-negative cultures may be more suitable for depyrogenation processes typically found in biopharmaceutical product streams. The cell wall fragments and outer membrane constituents associated with these endotoxins represent realistic challenges to process operations such as ultrafiltration, affinity chromatography, and the use of charged media membranes or columns.

Challenge studies for LPS or endotoxin removal in process streams should be conducted at the laboratory or pilot scale so as not to introduce high levels of endotoxin or LPS into the actual production environment.

4. PREPARATION AND USE OF ENDOTOXIN INDICATORS

4.1 Methodology to Create a Laboratory-Prepared Endotoxin: Principles to Consider

Glass vial EIs purchased from third-party vendors do not need further preparation before use. These indicators are labeled with a nominal value of inoculated LPS, and the label claim should be confirmed upon receipt to assure that there is sufficient activity (endotoxin unit, or EU) available for the study.

- There is not one "best" or "standard" method for preparing endotoxin in the laboratory, but one example of a published method for the preparation of laboratory-prepared endotoxin may be found in Bowers and Tran (*2*). Regardless of the methodology for preparation, the following recommendations should be considered to properly and consistently produce, identify, and maintain laboratory-prepared endotoxin for use as a tool for depyrogenation studies. An appropriate Gram-negative bacterial strain from a recognized culture collection is a good choice for preparing a laboratory-derived endotoxin. Alternatively, a Gram-negative organism isolated from a facility, water system, raw material, or product that is identified to the species level, that has been shown to be genetically stable and that is properly maintained, may also be considered. Establishing the identity and baseline genetic fingerprint of an environmental organism will assure that subsequent preparations are consistent.
- The laboratory should create detailed procedures or laboratory work instructions for culture maintenance and endotoxin preparation to assure consistency between batches of endotoxin. For example, endotoxin may be isolated from a culture of Gram-negative bacteria, according to the method of Bowers and Tran (*2*) or a well-documented variation of that method. Whatever the methodology for growth and endotoxin isolation that is developed by the laboratory, the methodology should be documented and used consistently.
- Consistent with good microbiological practice, the culture and maintenance of the cells used to produce a laboratory-prepared endotoxin should be consistent with *Microbiological Best Laboratory Practices* ⟨1117⟩. Instructions on 1) the proper maintenance of the organism; 2) growth conditions, including any requirements to prepare media, nutrient requirements, and time/temperature of incubation; 3) methods for cryopreservation or lyophilization for master cell banks and working cell banks; 4) storage of the endotoxin, once prepared including concentration, vessel type, and volume; and 5) master batch production records to assure consistency in subsequent studies should be written, managed via change control, and followed.
    - Once isolated, the relative activity of the endotoxin preparation should be established by comparing its activity to a known LPS standard such as RSE, or a CSE that has been standardized against the RSE. Determination of activity involves diluting the endotoxin preparation and assaying the dilutions against an LPS standard curve such that the result of the dilution falls within the range of the referenced standard curve. As with the CSE standard used in the bacterial endotoxins test (BET) assay from *Bacterial Endotoxins Test* ⟨85⟩, the activity of the endotoxin may vary, depending on the lot of lysate and lot of LPS used for the analysis. It is recommended, consistent with the assignment of potency for the CSE, that activity of an endotoxin preparation be evaluated for each lysate manufacturer, lysate lot, and test method (gel, kinetic turbidimetric, or kinetic chromogenic) in use in the laboratory.
      The activity of the stock endotoxin preparation in EU/mL is reported as:

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(Test result in EU/mL) × (dilution factor) = EU/mL of the starting endotoxin preparation

- Once activity has been determined, and if applicable to the study design, a standard series of dilutions of the newly prepared endotoxin should demonstrate onset times that result in slope and *y*-intercept values that are consistent with the standard curve parameters of the RSE/CSE standard using the same lot of lysate. This demonstrates that the activity of endotoxin preparation dilutes and reacts with the lysate in a manner that is similar to LPS.
- Characterization of the endotoxin preparation should also include data on the stability of the preparation, because stability is critical to the comparison of data from one study to the next. If the endotoxin preparation is stored, storage parameters including the concentration of the preparation in EU/mL, the composition of the vessel, the temperature of storage, and the length of storage, should be defined. An expiration date should be assigned based on determined stability.

4.2 Inoculation of EIs

To prepare an EI in house, inoculate endotoxin or LPS onto an article (carrier) that will serve as the substrate for the EI. Carriers for EIs can be anything that is subject to depyrogenation such as: vials (for dry heat depyrogenation), stoppers (for stopper washing), stainless steel coupons (for vessel cleaning), or product (for depyrogenation of process streams).

The simplest way to inoculate these indicators is to add a small volume of a highly concentrated solution of endotoxin or LPS to the carrier. The volume and concentration of added endotoxin or LPS should be calculated to add at least 1000 EU to the carrier, although higher or lower concentrations may be justified based on historical data on the endotoxin content of the material. [NOTE—The remaining discussion assumes a 1000-EU inoculum, but the principles hold for any level of initial inoculum.] For nonliquid carriers, the endotoxin is "fixed" or dried onto the carrier substrate. This fixing step is most easily accomplished by drying in a unidirectional air flow cabinet or hood, although other drying methods including vacuum drying, lyophilization, and other fixation methods could be used. In depyrogenation challenge studies, once a fixing method is chosen, it should not change in subsequent studies to assure comparability of results. Before using the EIs, a recovery procedure, consisting of a reconstitution or extraction method, should be developed and verified for consistency (*3*).

For liquid carriers such as bulk product, the level of inoculation in EU/mL should be justified based on "worst case" challenge for the depyrogenation step under study, meaning that the highest concentration of endotoxin that could be in the upstream product, based on process knowledge and historical endotoxin values, should be used. Such justification should take all contributing factors into account, including but not limited to: Gram-negative bioburden in raw materials and bulk; endotoxin content in raw materials including water, contributions by product contact surfaces; and the effect of hold times, particularly for nonsterile bulk.

4.3 Recovery of Endotoxin from EIs

To use EIs, it is necessary to recover and quantify the activity of the endotoxin or LPS from both unprocessed indicators (controls) and from processed indicators (i.e., those that have been through the depyrogenation process). LPS tends to adsorb to surfaces and may aggregate or disaggregate in some product matrices; therefore, recovery of activity from EIs made with LPS is often not 100% of the nominal or measured spike value. This section addresses the methodology for recovery and possible strategies for addressing recoveries that may be observed in challenge testing.

In the case of commercially available EIs prepared with LPS, the manufacturer's directions for extraction and recovery should be followed. With such products, there should be little difficulty in achieving recovery within a factor of 2 of the labeled LPS concentration. If recoveries within the specified range cannot be achieved, the manufacturer should be contacted for technical assistance.

For EIs made in-house using LPS, the composition of carriers, such as plastics, can affect recovery or result in inconsistent recovery because of adsorption. For these carriers, there is no prelabeled concentration to verify. In this case, the expected recovery should be based on the measure of the activity of endotoxin or LPS added to the article and the volume of extraction fluid used to recover it. The actual (measured) activity in the extract should then be compared to the measured activity of the endotoxin or LPS added to determine the percentage of recovery.

For example, consider a stock endotoxin or LPS preparation containing a measured activity of 100,000 EU/mL that is used to prepare in-house EIs. If a volume of 50 µL of this preparation is dried on the surface of each of a number of 10-mL vials, the known amount of activity added is 5000 EU. If the recovery/extraction is performed in 5 mL of water for BET, and the recovery is 100%, the expected activity in the extract solution is 1000 EU/mL.

$$\frac{5{,}000\ \text{EU/vial}}{5\ \text{mL extraction solution/vial}} = 1000\ \text{EU/mL}$$

If, however, the measured activity after extraction is 200 EU/mL as opposed to the expected 1000 EU/mL, the efficiency of the extraction method is 20%.

Recovery of endotoxin or LPS from nonliquid EIs prepared in-house can follow recommendations for the extraction of medical devices in preparation for LAL testing. *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩ states, "The standard extraction method is to soak or immerse the device or flush the fluid pathway with extracting fluid that has been heated to 37 ± 1.0°, keeping the extracting fluid in contact with the relevant surface(s) for NLT 1 h." The volume used for reconstitution or extraction should be appropriate for the material, size, and shape of the EI, recognizing that a volume too low may not efficiently recover the endotoxin or LPS and that excessive volumes will unnecessarily dilute the endotoxin or LPS that has been extracted.

If the recovery of added endotoxin or LPS is variable, an alternate extraction method may be developed and validated. This may include agitation or mixing, sonication or alternative extraction solutions. A combination of extraction in 0.01% sodium laurel sulfate, sonication, and vortex mixing is one such approach that has been reported to be more effective than extraction in water for medical devices (*4–6*). Other extraction methods are summarized by Bryans et al. (*7*) and in ANSI/AAMI standard ST72:2011 (*8*).

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Another situation concerns liquid endotoxin or LPS preparations that are used either to validate a depyrogenation process in a process stream or to investigate the destruction or removal of endotoxin or LPS in a manufacturing process. In these cases, the initial concentration of the stock liquid endotoxin or LPS solution should be measured before it is added to the system or process. If some of this preparation is added ("spiked") to a bulk process solution that is then subject to a particular process or treatment, the activity of endotoxin or LPS in this bulk solution should be measured and recorded as the starting activity. It is important to determine whether changes in the endotoxin or LPS activity of the processed solution are due to effects of the process and not to instability of the LPS or endotoxin in the solution. The stability of the activity of the LPS or endotoxin in these preparations should be verified over a period appropriate to the proposed use of the preparation.

As with the spiking method (choice of endotoxin/LPS and "fixing" process), whichever reconstitution/extraction procedure is chosen should be verified for consistency and should be used for all subsequent studies to assure comparability of results.

4.4 Choice of Test Methodology for the Analysis of EIs

Any of the test methods described in ⟨85⟩ can be used for the analysis of processed and unprocessed EIs. As with the rest of the methodology, it is highly recommended that an assay (kinetic turbidimetric, kinetic chromogenic, or gel clot assay) be chosen during method development and used consistently throughout the initial study and in subsequent studies to assure that data are comparable. The use of alternate assays is permissible, provided that they are validated to assure that they are equivalent to or non-inferior to the standard compendial assays.

5. ANALYSIS OF RESULTS OF DEPYROGENATION STUDIES

To evaluate the effectiveness of a depyrogenation process, the residual activity that is recovered from processed indicators is compared to the endotoxin or LPS activity of unprocessed controls. Typically, the $\log_{10}$ of the endotoxin or LPS activity measured for the processed EI (or solution) is subtracted from the $\log_{10}$ of the measured endotoxin or LPS activity of unprocessed control indicators. The result of the subtraction is the log reduction that is attributable to the depyrogenation process. If there are multiple controls and/or samples of processed material (and there usually are), the most conservative approach is to subtract the highest $\log_{10}$ concentration recovered from the processed EIs (or solution samples) from the lowest $\log_{10}$ unprocessed control endotoxin activity. For example:

- The activities in three unprocessed EIs are 1286, 1000, and 1532 EU/mL
- The activities in three processed EIs are 0.634, 0.512, and 0.496 EU/mL

The log reduction is calculated as:

$$\log_{10}(1000) - \log_{10} 0.634 = 3 - (-0.198) = 3.198 \text{ log reduction}$$

Historically, a ≥3-log reduction has been required by regulatory/compliance guidance. However, depending on the process and historical data, a 3-log reduction may be either excessive or inadequate. For example, for glass vials with a low or nonmeasurable endotoxin content upon receipt, the requirement to continually and repeatedly revalidate with an acceptance criterion of a 3-log reduction of the endotoxin spike of >1000 EU is excessive. Alternatively, a fermentation process with an endotoxin content of $>10^7$ EU/mL in the clarified culture supernatant will require more than a 3-log reduction to achieve safe levels of endotoxin in the drug substance or drug product. Additionally, given the sensitivity of BET assays, it may not be necessary to spike with 1000 EU to demonstrate a 3-log reduction. For example, if the assay sensitivity is 0.005 EU/mL, one may choose to spike with 50 EU and demonstrate an ultimate recovery in the test articles of less than 0.05 EU/mL, which calculates to greater than a 3-log reduction. In any event, the design of experiments including an appropriate specification for the log reduction of processed indicators and required test sensitivity to demonstrate the specified log reduction should be established and justified in a preapproved protocol for the study. The total reduction, of course, may be achieved over several steps in a purification process. Thus, the necessary reduction is often achieved additively over the course of multiple purification steps.

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⟨1229⟩ STERILIZATION OF COMPENDIAL ARTICLES

BACKGROUND AND SCOPE

This general information chapter provides an overview of the concepts and principles involved in sterilization (by various modes) of compendial articles that must be sterile. It includes information about supportive sterilization processes utilized in their preparation.[1] More detailed recommendations are presented in specific information chapters for each sterilization mode:

- *Steam Sterilization by Direct Contact* ⟨1229.1⟩
- *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩
- *Monitoring of Bioburden* ⟨1229.3⟩
- *Sterilizing Filtration of Liquids* ⟨1229.4⟩
- *Biological Indicators for Sterilization* ⟨1229.5⟩
- *Liquid Phase Sterilization* ⟨1229.6⟩
- *Gaseous Sterilization* ⟨1229.7⟩
- *Dry Heat Sterilization* ⟨1229.8⟩
- *Physicochemical Integrators and Indicators for Sterilization* ⟨1229.9⟩
- *Radiation Sterilization* ⟨1229.10⟩
- *Vapor Phase Sterilization* ⟨1229.11⟩

In the strictest definition of sterility, a specimen is deemed sterile only when there is a complete absence of viable microorganisms (bacteria, yeasts, and molds), but sterility cannot be demonstrated with respect to compendial articles and other items because of the inherent limitations of the current test (see *Sterility Tests* ⟨71⟩). Sterility, therefore, is defined in probabilistic terms that establish an acceptable level of risk. Sterility can be accomplished only by the use of a validated sterilization process under appropriate current good manufacturing practices and cannot be demonstrated by reliance on sterility testing alone. The basic principles for control of sterilization processes, including method development, validation, and ongoing assurance, are as follows:

1. Sterilization process development that includes evaluation of the stability and compatibility of materials, container integrity, expected presterilization bioburden, equipment method control parameters, etc.
2. Identification of sterilization process parameters that preserve the inherent properties of the materials yet inactivate or remove microorganisms.
3. Demonstration that the sterilization process and equipment are capable of operating within the prescribed parameters and corresponding to independent measurements of the critical parameters.
4. Performance of replicate studies that represent the operational range of the equipment and employ actual or simulated product. The use of biological indicators for correlation between the measured physical parameters and the expected lethality is recommended wherever possible.
5. Maintenance and monitoring of the validated process during routine operation.
6. Assurance that the bioburden (number and type) of the materials is acceptable and is maintained within predetermined limits during routine operation.

VALIDATION OF STERILIZATION PROCESSES

Validation of sterilization processes requires knowledge of sterilization technology and use of the appropriate instrumentation and equipment to control and verify critical sterilization process parameters. An important aspect of the sterilization validation program involves the use of biological indicators when appropriate. All sterilization processes should be maintained in a state of validation that includes periodic requalification. The validation program for each type of sterilization comprises several formally documented stages.

The general principles of validation programs are applicable to all sterilization procedures. Individual details are presented in the specific *USP* informational chapters for each sterilization mode.

The *process development* stage investigates and establishes the operating parameters that define the controls that will be used for the sterilization process. Portions of the cycle development can be performed in a laboratory setting. The *installation qualification* stage establishes that equipment controls and other instrumentation are installed as specified and calibrated. Documentation should demonstrate the acceptability of the required utilities such as steam, water, and air. The *operational qualification* stage confirms that the equipment functions within the defined sterilization parameters. The *performance qualification* stage of the validation program directly evaluates the sterilization of materials or articles. This determination requires independent parameter measurement during the sterilization process, as well as biological challenges in operational configurations. Correlation between the physical measurements and the demonstrated microbiological lethality or removal

[1] These processes may also provide depyrogenation, the extent of which depends on the actual sterilization conditions. (Depyrogenation by various means will be addressed in a chapter under development.)

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capability for sterilizing filtration methods supports the effectiveness of the sterilization process. The *routine process control* stage of the sterilization process requires a number of supportive practices and is outlined in detail below.

ESTABLISHING AND JUSTIFYING STERILIZATION PROCESSES THAT RELY ON MICROBIAL INACTIVATION

Articles intended to be sterile must attain a $\leq 10^{-6}$ probability of a nonsterile unit, i.e., less than or equal to one chance in one million that viable *bioburden* microorganisms are present. [NOTE—This is also called the Sterility Assurance Level. The term *Probability of a Nonsterile Unit* (PNSU) is used throughout this chapter because it is descriptive and substantially easier to understand.] This PNSU can be accomplished by balancing the method effect on the materials and the destruction of the bioburden (see *Figure 1*). Three methods are currently in use: overkill, bioburden/biological indicator, and bioburden-based methods. These methods are described in greater detail below. Regardless of the method chosen, the objective is a maximum PNSU of $\leq 10^{-6}$ for the bioburden. An overkill method is the simplest method to establish but has the greatest impact on materials. The bioburden-based method requires the most method control but subjects the materials to the least stressful conditions. Confidence in the process's lethality is the same, regardless of the method utilized.

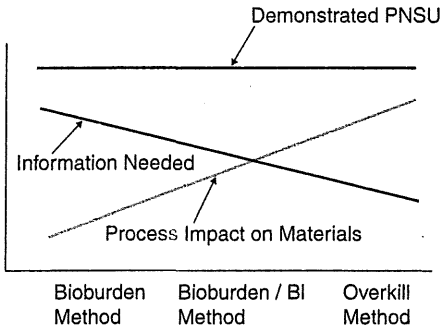

Figure 1. A basic comparison of various validation methods.

For items that are essentially unaffected by the sterilization process, the overkill method is preferred because of its simplicity. Overkill sterilization can be defined as a method in which the destruction of a high concentration of a resistant microorganism supports the destruction of reasonably anticipated bioburden present in routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum lethality; a defined set of method conditions; or confirmation of minimum log reduction of a resistant biological indicator.

If articles could be damaged by extended exposure to the sterilization process, it may not be feasible to employ an overkill method. In these instances, demonstrating the effectiveness of the sterilization cycle requires not only information about the delivered method conditions but also knowledge about resistance and control of the population of the materials' bioburden. This method is widely used for the terminal sterilization of heat-labile solutions and laboratory media. This sterilization strategy is variously called the bioburden/biological indicator or combination based method and is defined thus:

> Bioburden/biological indicator based sterilization is an approach in which the incomplete destruction (or destruction of a modest population) of a resistant biological indicator can be used to demonstrate the capability of the method to reliably destroy the bioburden present. This is accomplished using detailed knowledge of the bioburden and biological indicator populations and their relative resistance.

The bioburden-based method is used when material stability is limited or when there are no suitable biological indicator microorganisms available to use with the sterilizing process. Customarily, radiation sterilization is validated using the bioburden based method. The bioburden-based method can be defined as:

> A method in which bioburden samples from the material are routinely evaluated for resistance to the sterilization process and may be utilized to demonstrate the effectiveness of the process. Routine monitoring of the bioburden population and its resistance to the sterilization process is mandatory.

Filter sterilization of liquids and gases differs from other sterilization modes because filtration relies on removal of microorganisms from the fluid rather than inactivation by chemical or physical means.

D-Value and Microbial Resistance

The D-value is the time (customarily in minutes) or radiation dose (customarily in kGy) required to reduce the microbial population by 90% or 1 $\log_{10}$ cycle (i.e., to a surviving fraction of 1/10) and must be associated with the specific lethal conditions at which it was determined (see *Figure 2*). For steam and dry heat, the D-value is a function of temperature. In gas sterilization (ethylene oxide, ClO_2, or O_3), D-values are a function of the chemical concentration, relative humidity, and temperature. Similarly, for liquid chemical sterilization the D-value is a function of the temperature and sterilant concentration. [NOTE—Determining the D-value for vapor (condensing) systems such as H_2O_2, H_2O_2 plasma, and peracetic acid is complex because of the biphasic nature of the materials. Radiation and filtration sterilization are validated using unique methods. These processes are validated by methods that differ from those in this introductory chapter.]

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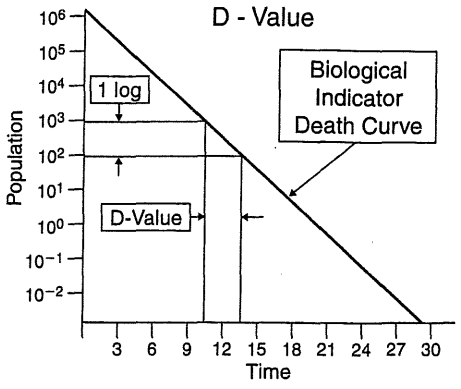

Figure 2. Graphical representation of D-value.

The D-value is not an inherent attribute of the microbe only, so the influence of other factors such as substrate, matrix, recovery media, and test methodology must be considered in D-value determination. The resistance of a biological indicator is defined for the indicator as a system. Accurate assessment and comparison between D-values requires standardization of test methods. To properly evaluate the effectiveness of a sterilization process, analysts must evaluate the resistance of the bioburden experimentally or via a literature search.

The death curve for microorganisms subjected to a sterilization process is comprised of three distinct regions (see *Figure 3*):

1. *Survivor curve region*—Where viable microorganisms can be recovered and counted to determine the slope of the death curve. Using survivor counts in short exposure periods, the first section of the death curve can be drawn to where the population is approximately 10 CFU.
2. *Fraction negative region*—Where replicate studies with multiple biological indicators are used to estimate the slope. This can extend the demonstrable portion of the death curve to approximately 10^{-2} to 10^{-3}.
3. *Estimated region*—Where the death rate curve established by either the survivor curve method or fraction negative method is extrapolated to the desired PNSU. Below 10^{-3} the death curve is assumed to be linear and is depicted in *Figure 3* by the dashed line beyond the point assuming that the death of microorganisms continues at the same rate.

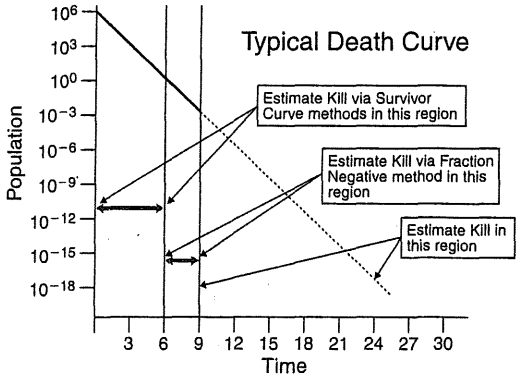

Figure 3. Death curve showing the various regions.

As stated earlier, the goal of the sterilization process is inactivation of the bioburden without adversely affecting product quality attributes. Demonstration of the lethality of a sterilization process under routine operation relies on differences in the relative resistance and population of the bioburden relative to the biological indicator (see *Figure 4*). Where the overkill method is used, bioburden controls can be less rigorous.

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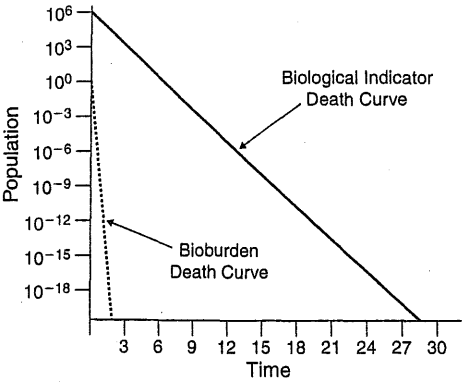

Figure 4. Relative resistance and population of typical biological indicator and bioburden microorganisms.

Validation of sterilization processes links physical measurements with biological indicator performance to establish method lethality. [NOTE—In radiation sterilization bioburden response is linked to physical irradiation dosage measurements.] Knowledge of the method's effectiveness coupled with bioburden controls on the materials under processing and information on bioburden resistance allow determination of the probability of a nonsterile unit.

Analysts must know the resistance of the biological indicator to the process in order to ensure that the organism's response to the process is properly understood. Equally important is an understanding of the bioburden present during routine use of the sterilization process and its possible resistance to the chosen process.

Biological and Physical Data

The biological indicator, when used, is a convenient means to simplify the sterilization validation effort. Biological indicators customarily are bacterial spores that have established resistance to the sterilization process under evaluation. When supplied as spores (on a substrate or as a suspension) with known initial population and resistance, their response to the method can be correlated to the measured physical conditions. Spores are preferable as biological indicators because their resistance and population are predictable and stable when they are handled, stored, and used as recommended.

The spores can be placed in the sterilization load in locations where physical parameter measurements such as temperature or gas concentration cannot be easily obtained (e.g., within needle lumens, syringes, and ampuls) or where measurement may alter the delivered conditions (e.g., sampling of the lethal gas). The biological indicator provides a means to directly assess the sterilizing effect of the method in a manner not possible by physical measurements. The lethality-based physical measurement and biological inactivation data from a sterilization process should be in reasonable agreement. When this is not the case, an investigation should be considered.

STERILIZATION INDICATORS AND INTEGRATORS

The execution of sterilization processes can be supported by physical and chemical indicators and integrators that provide an indication that processing is completed. These are available in many different forms for use in conjunction with many common sterilization processes.

Sterilization indicators respond to sterilization process parameters in a nonquantitative fashion; i.e., they show passing or failing results. They are useful in an operating environment as a means to identify whether an item has been exposed to a sterilization process. They are of minimal use in directly establishing process efficacy. Sterilization integrators are more sophisticated devices that react quantitatively in response to one or more of the critical sterilization parameters and yield a result that can be correlated to lethality. The most sophisticated integrators are radiation dosimeters that are so accurate and robust that their use has displaced the use of biological indicators for the validation of radiation sterilization. Additional detail about integrators and indicators can be found in *Physicochemical Integrators and Indicators for Sterilization* ⟨1229.9⟩.

STERILIZATION PROCESS DEVELOPMENT

An important consideration in any sterilization activity is the selection of an appropriate process from the many possible alternatives: steam, dry heat, gas, radiation, vapor, chemical, or filtration. The choice of the appropriate method for a given item requires knowledge of the sterilization process and information concerning effects of the process on the material being sterilized. The selection of a particular sterilizing treatment and the details of its execution often represent a compromise between the conditions required to destroy or remove the bioburden to the desired level and the impact of the sterilization process on the materials being processed. Sterilization processes should be sufficiently robust for certainty of microbial inactivation while avoiding adverse consequences to material quality attributes.

The overkill method employs conditions that are capable of destroying a high concentration of a resistant biological indicator and thus are a greater challenge to material integrity and stability. Overkill is employed only where the items being sterilized can withstand extended exposure to the sterilizing process and is used most commonly for metal, glass, and other items that are unaffected by process exposure. Its use is always preferable where materials can tolerate the more aggressive conditions utilized.

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The half-cycle validation method is a special case of the overkill method in which a lethal cycle to the biological indicator is arbitrarily doubled. Its use unnecessarily exposes materials to harsh conditions, and it should be avoided.

Bioburden/biological indicator (or combination) methods are appropriate when the product has some sensitivity to the sterilizing conditions. Analysts commonly use it for large- and small-volume parenterals, in-process solutions, and laboratory media for which the material properties would be impaired by a lengthy exposure to the sterilizing conditions. The proper use of the method requires control over the presterilization bioburden levels and confidence that the bioburden's resistance is such that it will be destroyed during processing. The complete destruction of or the use of a high population of the bioburden/biological indicator is not necessary for use of this method because it relies on differences in the relative resistance and population of the biological indicator and bioburden microorganisms.

The bioburden method bases the sterilization duration solely on the expected population and resistance of the bioburden on the materials. This is the method of choice for all radiation sterilization. It relies on periodic bioburden monitoring and resistance screening to establish confidence in the method. The bioburden method does not require use of a biological indicator.

Filtration is used for liquids and gases that either will not withstand heat, radiation, or chemical sterilization processes or are more conveniently sterilized in-line.

ROUTINE PROCESS CONTROL

After a sterilization process has been initially validated, it must be maintained in that state to ensure continued acceptable operation. This is accomplished by a number of related activities that are essential for continued use of the method.

Calibration—Equipment instrumentation must be periodically calibrated against a traceable standard. This includes recording as well as controlling instruments that regulate the operation of the equipment.

Physical Measurements—Data reported by the equipment sensors and recorders must be verified after the completion of each sterilization cycle. The records from the sterilization equipment are an essential part of the documentation.

Physical Integrators/Indicators—When they are used, integrators, and to a lesser extent indicators, provide an immediate indication of method execution and differentiate between processed and unprocessed materials. When these integrators provide a direct indication of method lethality (e.g., radiation dosimeters), they can be used for material release.

Parametric Release—The release of finished products without sterility testing is addressed in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩.

Additional Considerations—Depending on the specifics of the particular sterilization process, there may be additional requirements for confirming method efficacy. These can include bioburden sampling, bioburden resistance determination, biological indicator resistance determination, and supplier audits. As applicable, these activities should be carried out to maintain the sterilization process in a validated state.

Change Control—To maintain a validated state, materials, procedures, and equipment that influence the sterilization process should be monitored to ensure that all changes are recorded and evaluated in terms of their potential impact. To accomplish this, analysts must establish a formalized system for change control.

Preventive Maintenance—The user should establish a defined preventive maintenance program for each piece of sterilizing equipment in accordance with the equipment manufacturer's written recommendation. Preventive maintenance represents a special class of predefined changes that have no adverse effects on the operation of the system and thus do not require evaluation under change control.

Periodic Reassessment—In the absence of change to the materials, method, or equipment, the effectiveness of sterilization processes should be reconfirmed on a periodic basis. This system should be formalized and should address the potential impact of a number of de minimus changes or undetected changes to the validated system. In the absence of change, the amount of information required to support a sterilization process is less than that required for initial acceptance of the sterilization process.

Training—Sterilization processes rely heavily on scientific principles for the effective destruction of microorganisms. Scientists and engineers well-grounded in the principles of microbial death and removal develop processes to ensure effective sterilization. Individuals involved in the development of sterilization processes require a background in microbiology, physics, chemistry, and engineering, and they must be familiar with good manufactoring principles and regulations. Sterilization is an interdisciplinary activity where the combined knowledge of a group of individuals is generally required for the establishment of a reliable process. In addition to the sterilization process development team, individuals responsible for maintenance and operation of sterilization processes must also be trained appropriately to ensure that their actions contribute to success. These individuals are often the first to identify upsets and shifts in process performance because of their intimate involvement with it. Effective training programs should be established and documented. Training programs should emphasize sterilization principles, adherence to established processes and procedures, and the importance of documenting deviations from normal operations.

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⟨1229.1⟩ STEAM STERILIZATION BY DIRECT CONTACT

SCOPE AND BACKGROUND

Steam sterilization is perhaps the most common of all sterilization processes. It is used in settings ranging from practitioner offices to large-scale manufacturing facilities. The diversity of practices that use steam sterilization is reflected in the range and sophistication of the equipment used. This general information chapter addresses sterilization in which saturated steam comes into direct contact with the load items (whether wrapped or unwrapped) and provides an overview of the basic concepts of this mode of sterilization, including its validation. The load items in this sterilization process are variously termed parts, components, hard goods, wrapped goods, or porous goods. These items may be metallic, glass, ceramic, elastomeric, or polymeric materials that have little or no sensitivity to thermal degradation at the sterilizing temperatures. For steam sterilization by direct contact, it is customary to sterilize items using an overkill method.

Sterilization of liquid-filled containers may be substantially different. *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩ provides information about applications in which steam is a heating medium but is not in contact with the sterilization target, the liquid in the container.

SATURATED STEAM

Saturated steam is a biphasic mixture of H_2O in gas and liquid phases in thermal equilibrium. Saturated steam has a singular temperature–pressure relationship in which both phases are present, and at a given temperature only one pressure is possible for saturation. The importance of using saturated steam for sterilization arises primarily from two attributes. First, saturated steam rapidly kills microorganisms because of the presence of liquid water. Steam heated above saturation, also termed superheated steam, lacks liquid water, and although it is higher in temperature than saturated steam it is substantially less lethal to microbes. Second, when steam changes phase from gas to liquid, it releases thermal energy (2202 kJ/kg at 121°) that is transferred to the load items, facilitating sterilization of their exposed surfaces.

The initial objective for saturated steam sterilization is that the air in the sterilizing chamber must be replaced by saturated steam. Residual air within the sterilizer chamber and load items acts as both an insulator and an obstacle to steam penetration to all surfaces of the load items, and its removal is essential for effective sterilization. The presence of residual air in the chamber negates the singular temperature–pressure relationship of saturated steam. In the absence of saturation, physical measurements may not provide assurance of lethality.

GRAVITY DISPLACEMENT CYCLES

In the simplest autoclave cycles, air removal is accomplished by gravity displacement. Because steam is hotter and less dense than air, it rises to the top of the autoclave, and the colder air exits at the bottom of the chamber. Saturated steam entering the chamber changes into liquid condensate as it contacts the colder surfaces of the autoclave chamber and load items. Retention of condensate within the load reduces cycle effectiveness because it is a barrier to steam contact, and additional steam is needed to maintain the saturated steam at the sterilizing temperature. The load items, wrapping materials, and load arrangement should be designed to facilitate air removal and condensate drainage. In gravity displacement cycles, the load slowly reaches the desired sterilizing temperature because air removal is relatively slow compared to cycles in which its removal is mechanically assisted. During the exposure segment of the cycle, a thermostatic trap at the bottom of the chamber drain allows the removal of condensate (and any residual air) from the sterilizer while maintaining sterilizing conditions. At the conclusion of the dwell period, the chamber is returned to atmospheric pressure.

PREVACUUM CYCLES

To remove air more effectively from the chamber and the load items, sterilizers may employ multiple evacuation/pressure pulses in which air is replaced by steam. The number and depth of these pulses may vary. Because the alternating vacuum and pressure pulses may stress wrapping materials, the latter must be chosen carefully. The operation of the sterilizer during the exposure segment is similar to that of the gravity displacement cycle previously described. The vacuum system can be used at the end of the process to remove residual steam and condensate from the load items. The selection of a specific cycle and its associated sterilization parameters for a given item depends on a number of factors, including the heat lability of the material, heat penetration into the article, the item's mass, difficulties with air/condensate removal, and other factors described in the validation program (see below).

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STERILIZATION CYCLE CONTROL

Sterilizers are controlled by computerized/automated systems that manage the overall process execution and data reporting. The systems for steam sterilization may be controlled by calibrated temperature and/or pressure sensors on the equipment. During the exposure portions of the cycle, a minimum dwell time at a predefined temperature is required to ensure the method lethality target (minimum time–temperature or F_0) is met. Cycle efficacy for steam sterilization often is measured using F_0, which is defined as the equivalent exposure time at 121°. F_0 is a means for quantifying steam sterilization effectiveness by determining the equivalent sterilization time in minutes relative to a base temperature of 121° and a z-value of 10°; z-value is defined as the number of degrees of temperature change necessary to change the D-value by a factor of 10. The F_0 method is used to evaluate sterilization processes operated at varying temperature conditions to a single standard.

The process lethality at temperatures other than 121° can be calculated to determine lethality equivalent to that provided at 121°. Moist heat sterilization process efficacy is not intrinsically linked to a target temperature of 121°, which is simply the Celsius conversion of 250°F, and other temperatures can be used. Sterilizer control systems for direct sterilization typically provide a minimum time at a defined set point temperature after the initial air/condensate removal. Steam sterilizers are controlled using temperature sensors located in the drain line before the thermostatic trap, although other control schemes may be used. The temperature at this location typically is recorded for permanent documentation of sterilizing conditions. In sterilization by direct contact, exceeding the minimum time–temperature requirements or F_0 is acceptable because of minimal adverse consequences to the materials being sterilized.

Total lethality can be calculated over the course of the process. For the specific reference temperature of 121° and a z-value of 10°, the total accumulated F_0 can be determined by the following equation:

$$F_0 = \int_{t_1}^{t_2} 10^{\left(\frac{T-121}{10}\right)} dt = \sum_{t_1}^{t_2} 10^{\left[\frac{T-121}{10}\right]} \Delta t$$

where

t_1 = start time

t_2 = end time

T = temperature

Summing the instantaneous lethality contributions over the entire sterilization process allows the calculation of the overall process lethality or F_0 delivered. The F_0 calculation should begin at 100° and should continue through the end of the dwell period provided that saturated steam conditions are maintained.

VALIDATION OF STERILIZATION BY DIRECT CONTACT

The predominant approach for steam sterilization by direct contact is the overkill method defined in *Sterilization of Compendial Articles* ⟨1229⟩. Overkill sterilization is a method in which the destruction of a high concentration of a resistant microorganism is correlated with the destruction of reasonably anticipated bioburden present during routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum lethality (F_0), a defined set of physical conditions, or confirmation of a minimum log reduction of a resistant biological indicator.

The validation requirements for the overkill method are less onerous than those for other methods such as those based on bioburden or bioburden/biological indicators. When the load items can withstand substantial heat without adverse consequence, overkill is the method of choice for steam sterilization because of its ease of execution, reduced considerations for bioburden control, and overall simplicity.

Equipment Qualification

Equipment qualification is a predefined program that examines the equipment to confirm that it has been properly installed and operates as intended before the sterilization process. Equipment qualification can be separated into installation qualification and operational qualification, or can be considered joint installation and operational qualification. The qualification effort provides a baseline for the sterilizer's preventive maintenance and change control.

Empty Chamber Temperature Distribution

A common procedure to evaluate steam sterilizer installation is the evaluation of the empty chamber's performance. Each air removal method used in the sterilizer is evaluated by temperature measurement near the corners of the sterilizer chamber, near the controlling probe, and other locations as appropriate. The distribution of temperatures in the empty chamber should be determined only by sensors located in the chamber, and the temperatures of the chamber drain or outside the chamber proper are not directly relevant in this validation activity. Differences in the cycle dwell period can be ignored because only the shortest dwell period for each air removal method must be evaluated. The acceptance criteria for this test vary with the sterilizer's capabilities and customary use. Biological indicators are not required in the evaluation of empty chamber temperature distribution.

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Component Mapping

Items that are steam sterilized can be quite complex and may have interior void volumes, obscured surfaces, crevices, and difficult-to-reach product contact surfaces that must be sterilized. The ability of saturated steam to penetrate the wrapping materials or containers and to reach the surfaces should be established for each item. Although this is relatively easy for simple items such as spatulas, beakers, and other simple geometric shapes, it can be substantially more difficult for filling assemblies, filter housings, tubing, and hoses. Analysts should conduct studies to determine cold spots in items to ensure that heat penetration takes place throughout the load items using thermocouples in contact with the item's surface. These studies can be performed in a laboratory setting and need not be repeated when the same item is sterilized in multiple autoclaves. During this evaluation, all load items should be wrapped and oriented in a manner that facilitates steam ingress and air and condensate removal. Items must be wrapped and oriented in an essentially identical manner for reproducible sterilization.

Load Mapping

The determination of loading patterns is an essential practice for terminal sterilization of aqueous liquids by moist heat (see *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩), but this practice is not a critical concern regarding direct sterilization of items because differences between components play a greater role than location within the load.[1] Loads for direct steam sterilization can be validated using a maximum and minimum load as determined by either the number of each item or their mass. Best practices include placing larger items on the lower shelves, allowing condensate from these items to exit the sterilizer with minimal contact with other load items.

Biological Indicators

The commonly used biological indicator for steam sterilization by direct contact contains spores of *Geobacillus stearothermophilus* (ATCC 12980 or ATCC 7953), a thermophilic microorganism with a moist heat resistance substantially greater than that of most vegetative microorganisms. The spore challenge can be placed on a substrate within or on a load item, or the challenge can be a load item that is inoculated with a spore suspension. When biological indicators are used according to the manufacturer's directions, the resistance information provided by the vendor can be used. End users must determine the population and resistance of inoculated items they prepare.

Heat Penetration and Microbiological Challenge

The goal of the validation activity is the confirmation of acceptable heat penetration using temperature measurements and biological indicator challenges. Customarily this study is performed under conditions where the exposure time and/or temperature are reduced slightly from the routine set points. Thermocouples and biological indicators should be placed with load items at the locations determined to be most difficult to heat during component mapping. Thermocouples should be in contact with the item's surface. Analysts must take care in the insertion of thermocouples and biological indicators so they do not alter the ability of the steam to enter the objects being challenged. This difficulty can be overcome with special fittings for thermocouple entry or by placement of temperature probes in units placed near the units that contain biological indicators. In the latter case, replicate studies provide proof of cycle efficacy when both the biological indicators are killed and the physical measurements correspond to the expected time–temperature values or F_0. If the microbial and physical measurements do not meet predefined acceptance criteria, an investigation is required and corrective action is necessary to rectify the discrepancy.

Routine Process Control

As with all sterilization processes, after validation, steam sterilization must be subject to formal controls that maintain it in a validated state over time. *Sterilization of Compendial Articles* ⟨1229⟩ outlines the general requirements for all sterilization processes including training, calibration, physical measurements, physical integrators or indicators, ongoing method control, change control, preventive maintenance, and periodic reassessment.

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⟨1229.2⟩ MOIST HEAT STERILIZATION OF AQUEOUS LIQUIDS

INTRODUCTION

Steam sterilization of aqueous liquids (including both suspensions and emulsions with mixing), also known as sterilization of nonporous loads, is the method of choice for aqueous parenteral products, in-process aqueous liquids, laboratory media, and biological waste materials. This type of sterilization is accomplished primarily in closed containers. During steam sterilization by direct contact (also called steam sterilization of parts, hard goods, or porous items) the steam in the chamber directly contacts the surface of load items to effect sterilization (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩). In contrast, sterilization of liquids in containers is accomplished by application of heat to the container, heating of the container wall, and ultimately heating of the internal liquid volume. This can be accomplished using steam, superheated water, and air in various combinations. Some aqueous liquids are susceptible to over-processing that could render them unfit for their intended use. Manufacturers should consider the influence of these differences when they establish a suitable process.

During the sterilization of liquid-filled containers, differential pressures between the interior of the containers and the sterilization chamber may potentially impact container integrity. Air over-pressure is used to minimize the pressure differential between the container and the sterilizer to protect the integrity of the container, especially prefilled syringes and plastic containers. Before sterilization of product containers, manufacturers should consider the potential adverse consequences of excess heat on the materials. In order to ensure sterility as well as functionality, the process definition and validation method used must incorporate both lower and upper temperature and time limits on the process conditions.

When the overkill method can be used for sterilization of sealed liquid containers, it is the preferred method and is described in *Steam Sterilization by Direct Contact* ⟨1229.1⟩. When product quality attributes can be impaired by excessive heat, the sterilization process should use less time or a lower temperature to minimize the adverse effect on the materials. Sterilization time–temperature or F_0 conditions (F_0 is defined as the equivalent sterilization time relative to a base temperature of 121°) include both lower (sterility-related) and upper (stability-related) limits.[1] Manufacturers commonly employ the bioburden/biological indicator (BB/BI) or bioburden methods when constraints on the material's ability to withstand the process require the use of less aggressive conditions. This approach requires appropriate controls on presterilization bioburden and/or product-related D-values in conjunction with bioindicators of lower spore count or resistance to ensure sterilization.

Terminal Sterilization of Products

The maintenance of product attributes may require the use of sterilizing conditions that are less aggressive and sterilization equipment, cycles, and validation methods adapted to these more restricted circumstances. The substantial variations in equipment designs and methods for terminal sterilization preclude a singular description of a typical cycle. All terminal sterilizers heat the load, but they accomplish this in varying ways: saturated steam; steam–air mixtures, steam–air–water mixtures, and superheated water. Air over-pressure for maintenance of container integrity and cooling containers and water for heating/cooling of the load may be present depending upon the autoclave size, throughput expectations, and container.

In-Process Fluids

In-process fluids are used for pH adjustment, dilution to a specified volume, lubrication, and other purposes. In many instances these liquids are sterilized in conjunction with items that must be sterilized by direct steam contact, and the sterilization process must ensure that all items are adequately sterilized.

Laboratory Media

Laboratory media often are sterilized in standard steam autoclaves with minor adaptations. Provision for slow exhaust (to reduce stress on container integrity and minimize boil over) and jacket cooling can help improve the basic steam sterilizer design and operation to better accommodate the materials. The sterilization process may be specific for media containers or a combination of both liquid-filled containers and hard goods. This process may resemble the methods used for terminally sterilized products (see above). The sterilization of laboratory media may entail the processing of a number of different containers that contain different materials. Manufacturers should be aware of the potential for under- and over-processing across the load and must consider container size, container contents, and position. When liquid-filled containers are combined in the same load with hard goods, manufacturers must consider the unique concerns of each to ensure all items are properly sterilized. Because laboratory media are considered self-indicating with respect to sterility, the use of internal biological indicators during validation is not required.

Biowaste Sterilization

The sterilization of biowaste in sealed containers from laboratory or production use is similar to parts sterilization. The process is defined to ensure a minimum time–temperature exposure or attainment of a specified F_0 value throughout all items of the load. Depending on the potential contaminants present, the autoclave design may incorporate condensate collection/sterilization or sterilizable exhaust filters to ensure that pathogens are adequately contained. Because the objective of biowaste

[1] Degradation kinetics may differ from those of microbial kill, and F_0 values may not be sufficient to fully evaluate "worst case" effects.

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sterilization is to render the materials safe for contact and disposal, the overkill method described in *Steam Sterilization by Direct Contact* ⟨1229.1⟩ is employed.

BIOBURDEN/BIOLOGICAL INDICATOR METHOD

Application of the BB/BI method requires a thorough understanding of the bioburden type, population, and resistance typically present in the presterilized product-filled container. The method relies on substantial differences between moist heat resistance and the population of the bioburden present and the biological indicator used during validation (*Figure 1*).

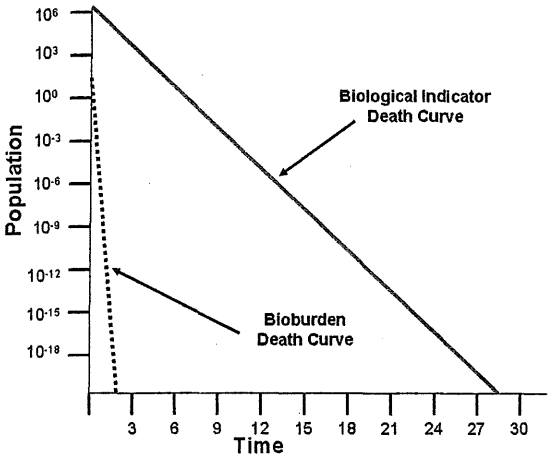

Figure 1. Relative resistance and population of typical bioburden and biological indicator microorganisms.

BB/BI is a method in which the incomplete destruction (or destruction of a modest population) of a resistant biological indicator can be used to demonstrate the capability of the process to reliably destroy any bioburden. This is accomplished using detailed knowledge of the BB and BI populations and their relative resistance.

Typical BB microorganisms have only minimal resistance in comparison to BIs, and this can be confirmed by heat screening of BB isolates. The BB population is controlled by filtration steps for the fluid, process time limits, environmental controls, gowning systems, and other means. The conventional BIs for terminal sterilization using the BB/BI method are *Clostridium sporogenes* ATCC 7955 and *Bacillus subtilis* ATCC 5230, although other strains can be used. The use of *Geobacillus stearothermophilus* for terminal sterilization is uncommon with the BB/BI method because the organism's strong resistance to moist heat makes it poorly suited for this application.

Confirmation of an acceptable probability of a nonsterile unit (PNSU) can be accomplished using physical measurements and BI response (which define the lethality of the process) in conjunction with processing limits for the BB population and resistance (which define the N_0 and D-value). D-value is the time (customarily in minutes) required to reduce the microbial population by 90% or 1 $\log_{10}$ cycle (i.e., to a surviving fraction of 1/10) and must be associated with the specific lethal conditions at which it was determined. For example, D_{121} is the D-value at 121°. Articles intended to be sterile must attain a $\leq 10^{-6}$ PNSU, i.e., less than or equal to 1 chance in 1 million that viable bioburden microorganisms are present. The PNSU can be determined from Equation 1.

$$\log N_u = -F/D + \log N_0 \quad [1]$$

N_u = PNSU
D = D-value of the natural bioburden
F_0 = F_0-value of the process (lethality)
N_0 = bioburden population per container

The following example indicates the resulting PNSU under the defined conditions of validation and routine operation (*Table 1*).

Table 1. Examples of PNSU Calculation

Validation	Routine Usage
F_0 = 8.0 min	F_0 = 8.0 min
D_{121} of BI = 0.5 min	D_{121} of bioburden = 0.005 min
N_0 of BI = 10^6	N_0 of bioburden = 100 (or 10^2)
PNSU for BI = 10^{-10}	PNSU for BB = 10^{-1598}

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Determining the resistance of the bioburden is accomplished using a heat-screening process during which a pure culture (a laboratory culture containing a single species of organism spores with minimal vegetative cells) is boiled at 100° for various periods. If the bioburden microorganism is viable after exposure, its resistance at 121° can be estimated for use in the PNSU calculation (*Table 2*).

Table 2. D_{121} Estimation from Boil Test Results

Exposure Time	Approximate D_{121}-value
1 min	0.01 min
10 min	0.1 min
20 min	0.2 min
100 min	1.0 min

Bioburden Method

In most respects the BB method is similar to the BB/BI indicator method. The difference lies in the isolation and characterization of the most-resistant bioburden microorganism. The worst-case isolate is used as the biological indicator in the evaluation of the process. For use in this manner, it must be cultured to produce a suitable challenge population. When this method is used, the bioburden of each process cycle must be closely controlled with respect to population and must be monitored for resistance.

Sterilization Cycle Control

Process equipment for terminal sterilization typically is controlled by calibrated and pressure sensors in/on the chamber/equipment. During the exposure portions of the cycle, attainment of a minimum dwell time at a predefined temperature is used to support process lethality. Cycle lethality for terminal sterilization customarily is measured using F_0, which is defined as an actual exposure time at a variable process temperature that is equivalent to an exposure at 121° based on an ideal microorganism with a z-value of 10°. This can include lethality delivered during the heat-up and cooling phases of the sterilization process. A *z*-value is defined as the number of degrees of temperature change necessary to change the D-value by a factor of 10. The F_0 approach is used to evaluate to a single standard sterilization processes that are operated at varying temperature conditions. The process lethality at temperatures other than 121° can be calculated to determine lethality equivalent to that provided at 121°. Sterilizer control systems for terminal sterilization deliver conditions within a predefined time–temperature or F_0 range to avoid over-processing.

Simple mathematics can be used to calculate the total lethality over the course of the process. For the specific reference temperature of 121° and a z-value of 10.0°, the F_0 calculation can be determined by Equation 2:

$$F_0 = \int_{t_1}^{t_2} 10^{\left(\frac{T-121}{10}\right)} dt = \sum_{t_1}^{t_2} 10^{\left(\frac{T-121}{10}\right)} \Delta t \qquad [2]$$

t = time

T = temperature

Summing the instantaneous lethality contributions over the entire sterilization process allows the calculation of the overall process lethality or F_0 delivered over the course of the entire process at varying conditions.

Validation of Liquid-Filled Container Sterilization

As previously noted, the preferred method for steam sterilization is the overkill method as defined in *Sterilization of Compendial Articles* ⟨1229⟩ and *Steam Sterilization by Direct Contact* ⟨1229.1⟩. However, when the processed materials are susceptible to damage by moist heat at the overkill conditions, the BB/BI method is better suited because it results in reduced heat input while affording the same degree of process efficacy but with different controls. As noted above, terminal sterilization processes require greater consideration of the effects of the treatment on material properties. This has implications for many of the elements of the qualification and validation exercises as indicated below. The validation requirements for the BB/BI and BB methods are more rigorous than those associated with the overkill method. Although the overkill method can be confidently used without detailed consideration of the presterilization bioburden, application of the BB/BI and BB methods require continued monitoring and control of the bioburden, specifically the population and resistance. This is accomplished by testing of filled containers just before sterilization and measuring the number of colony-forming units per container and confirming the absence of resistant BB isolates. When resistant isolates are found, their thermal resistance in the fluid should be determined.

EFFECT OF THE STERILIZATION PROCESS

A preliminary determination of the liquid and the container–closure system's ability to withstand the expected sterilizing conditions should be made during product development. This can be accomplished by sterilization at conditions slightly in excess of the maximum expected and evaluating the effect on the material. The evaluation should encompass the essential quality attributes with attention focused on known and potential new impurities. Appearance and other physical properties also should be evaluated as a part of this effort.

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EQUIPMENT QUALIFICATION

Equipment qualification is a predefined program that confirms the equipment has been properly installed and that it operates as intended. Qualification of the sterilizing equipment provides a baseline for preventive maintenance and change control for the sterilizer. The sterilization equipment may require qualification of air, water, utility, and other systems that impact the sterilization equipment's performance.

EMPTY CHAMBER TEMPERATURE DISTRIBUTION

The dual considerations of sterility and stability commonly associated with sterilization of liquids require that equal attention be paid to potential under- and over-processing of the load. For this reason the temperature gradient across the sterilizer may require substantially tighter control than that expected in sterilization by direct contact. The objective is to minimize the time–temperature or F_0 differences across the load throughout the process. Biological indicators are not required in the evaluation of empty chamber temperature distribution.

BIOLOGICAL INDICATORS

The selection of a BI must be considered carefully because of the balance that must be maintained between attaining sterilization and maintaining the sterilized material's essential quality attributes. The biological challenge is either directly inoculated into a liquid-filled container or is introduced via self-contained units provided there is adequate correlation between their resistance and the resistance that would occur in the process fluid. The liquid can be either the product or a surrogate fluid. The resistance of the indicator in the product (and surrogate fluid, where used) must be known. The surrogate's physical properties should approximate those of the product. If there are surfaces within the container that are not presterilized, biological challenge of those surfaces may be required.

LIQUID D-VALUE DETERMINATION

Determination of the thermal resistance (D-value and z-value) for the biological indicator in the liquid is required. This must be performed in a Biological Indicator Evaluation Resistometer (BIER) in replicate. The thermal resistance of each BI lot in the liquid should be determined. When a surrogate liquid is used for convenience (e.g., a master solution approach) or because of microbial inhibition of the BI by the liquid, the thermal resistance in the surrogate must be determined.

CONTAINER MAPPING

Liquid-fill containers with volumes greater than or equal to 100 mL should be mapped to determine internal cold spots. The mapping should be performed with product containers oriented as they would be within the load. The temperature probes should be introduced into the containers using methods that maintain container integrity. Internal supports (of minimally heat-conductive materials) may be required to ensure proper positioning of the probe within the container. After these locations are determined, they are used as either monitoring locations or are correlated to external conditions on the container during validation and routine processing. Smaller containers (less than 100 mL) have fewer discernable cold spots, the importance of which is reduced as container size decreases. Smaller containers (less than 100 mL) can be monitored with temperature probes secured to their exterior. The "cold spot" should be considered a "region" and not a single point in the container.

LOAD POSITIONING AND MAPPING

A fixed loading position within the sterilizer may be necessary for proper sterilization to ensure uniformity of heating and cooling in routine use. Once the load is positioned properly, its size can vary within a defined range. Load-mapping studies should be performed to determine the coldest and hottest locations within the load. These locations may not be specific individual containers but rather regions. This ensures that the containers are neither under- nor over-processed in routine operation of the sterilizer. Validation of variable-size load patterns is accomplished using a bracketing approach for which success with maximum and minimum loads (avoiding both under- and over-processing) establishes the acceptability of intermediate-size loads. However, evaluation of intermediate load sizes may be beneficial. In product sterilization, only a single-size container with a single product lot is processed concurrently.

HEAT PENETRATION AND MICROBIOLOGICAL CHALLENGE

The core of the validation activity is confirmation of acceptable heat penetration using temperature measurements and microbial challenge inactivation. Temperature probes and biological indicators are placed within the load at worst-case locations (e.g., the coldest portions of the loaded chamber). Introduction of the thermocouples must not alter the integrity of the container. Biological challenges are placed in containers adjacent to those that contain heat penetration probes (or the same unit with external temperature measurement).

Proof of cycle efficacy is provided by replicate studies in which the BIs perform as expected and the physical measurements correspond to the expected values of time and temperature or F_0. If the microbial and physical measurements do not correlate, an investigation is in order, and corrective action must be taken to rectify the discrepancy. Samples from the hottest regions of the load are used for evaluation of material stability and quality.

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PRODUCT QUALITY AND STABILITY EVALUATION

Manufacturers must conduct ongoing evaluation of the product's ability to withstand the routine sterilizing conditions. The evaluation should encompass the essential quality attributes with attention focused on known and potential new impurities and those materials that receive the most heat input. Manufacturers also should evaluate appearance, other physical properties, and container–closure integrity as a part of this effort. For microbiological media, the ability of the media to meet growth promotion and other requirements is required as indicated in the appropriate test chapter(s) (e.g., *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Sterility Tests* ⟨71⟩).

Routine Process Control

All sterilization processes should be subject to formalized practices that maintain them in a controlled state. The practices outlined in *Sterilization of Compendial Articles* ⟨1229⟩ include the general requirements appropriate for all sterilization systems. This is accomplished by a number of related practices that are essential for continued use of the process over an extended period of time. The practices include: calibrartion, physical measurements, physicochemical integrators, indicators for sterilization, monitoring of bioburden, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

The use of parametric release is common in the terminal sterilization of finished product containers. This subject is addressed in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩.

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⟨1229.3⟩ MONITORING OF BIOBURDEN

INTRODUCTION

Monitoring of in-process bioburden of pharmaceutical components and products is an essential element of the overall contamination-control program for appropriate sterilization process control. Bioburden monitoring should be designed for the recovery of a broad range of microorganisms that are likely to be present in the material being processed. Sterilization processes are implemented in order to eliminate bioburden in materials and the products, ensuring both adequate process control and end-user safety.

Bioburden is a potential risk to the patient not only because the sterilization process might not be completely effective, but also post-processing because of the possible presence of residual materials such as allergens, endotoxins, and exotoxins. It may also have adverse impact on product quality and stability. Therefore, although bioburden may be confidently killed by destructive sterilization processes or removed by retentive processes (filtration), as summarized in the ensuing sections, its proliferation before sterilization should be avoided. Process controls and cleaning, sanitization, and disinfection programs provide active means for the control of bioburden population and support the sampling, enumeration, and characterization of bioburden necessary to assure that sterilization processes are effective.

Destructive Processes

Destructive sterilization processes, e.g., moist and dry heat sterilization, are developed and validated to kill microorganisms. It is critically important to the quality control aspects of the sterilization program to fully understand the bioburden. The microorganisms that are most resistant to widely used destructive processes are spores of certain Gram-positive bacteria (note that mold spores are less resistant to these destructive processes than are bacterial spores). As a result of the resistance properties of these organisms, some species are commonly used as biological indicators for the evaluation of sterilization process performance. In their vegetative state, microorganisms that form spores do not exhibit extraordinary resistance to sterilization processes.

Processes that rely on ionizing radiation are an exception to typical resistance profiles because some non-spore-forming microorganisms exhibit higher resistance than do spore formers. Bioburden within materials and products subjected to radiation processes is evaluated as part of dose setting activities and is explained in the *Radiation* section that follows.

The sterilization process, the development of a thorough understanding of the process, and its microbiological impact are major elements in confirming that an acceptable level of sterility assurance is established. Understanding the inactivation kinetics of the typical bioburden as well as other potential bioburden microorganisms is critical to successful implementation. The new process should have a documented risk assessment outlining its monitoring and control aspects. The establishment of a new sterilization process requires the evaluation of the suggested biological indicator resistance (*1,2*).

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Retentive Processes

The microorganisms least likely to be retained by a sterilizing filtration process are those that are potentially smaller than the smallest pores in the filter matrix. Although size exclusion is an important factor, filters do not retain microorganisms solely by sieve retention. Adsorption, wherein microorganisms are retained within the filter matrix by entrapment or electrostatic forces, also is an important retention mechanism. The control of prefiltration bioburden is an important risk-mitigation factor in retentive processes, and this is particularly true when adsorption may be a significant retention mechanism. Bioburden removal capability therefore depends on the size and number of the bioburden microorganisms, the pore size distribution of the filter, the properties of the solution being filtered, and the filtration process parameters.

MONITORING AND SAMPLING

Statistical and analytical limitations complicate the evaluation of bioburden from both liquid and solid materials. Many products are inherently antimicrobial, and some formulations contain antimicrobial preservatives, both of which can limit bioburden recovery. Products that are outside of the pH range of approximately 4–9, are strongly hypertonic or strongly hypotonic, or have low water activity, may reduce the level of recoverable microorganisms.

The collection of multiple samples from the same bulk often gives varying results due to temporal and spatial differences in microbial distribution. Samples from varied components and different formulations will give different results because of different nutritional and cultural requirements, environmental stress to microorganisms, sampling methods, sample size, sampling pattern, species heterogeneity, and shifting microbial populations. Because of the technical challenges associated with bioburden analysis, results will be dependent upon sample-specific and method-specific variables when attempting to recover, grow, or enumerate organisms. Sampling frequency and maximum time delay between sampling and testing should be established based on previous data.

Presterilization bioburden analysis should be conducted on samples that are representative of materials produced during routine preparation and processing. Sampling frequency should be established based on previous data, known variability, batch size, material, process, and environmental influences. Bioburden should be recovered and enumerated from samples that are representative of the process material. When the material is stored in separate containers, analysts should consider testing multiple or composite samples. Bioburden evaluation should focus on microorganisms that represent a greater concern in the sterilization process. Total count methods should consider the properties of both the product under evaluation and the characteristics of the process that may affect recovery. Cultivation methods, diluents, and media selection must be based on past experience with the manufacturing process and test material (*3*). The methods listed in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩ and *Mycoplasma Tests* ⟨63⟩ may be appropriate for bioburden evaluation, although they may require modification to the methods and qualifications in order to meet the requirements of specific test materials.

Bioburden Screening

Materials and products that are to be sterilized should be examined to determine the level of bioburden in the article. Determining the microbial population level is an essential element in ensuring process efficacy. For lethal processes other than radiation, the presence of Gram-positive spore-formers represent the greatest potential for survival. The potential resistance of the bioburden to the specific sterilization process is an important consideration and can be evaluated by a screening process. Isolates that are identified as Gram-negative rods have the potential to cause high levels of endotoxin. *Figure 1* illustrates one possible bioburden screening program.

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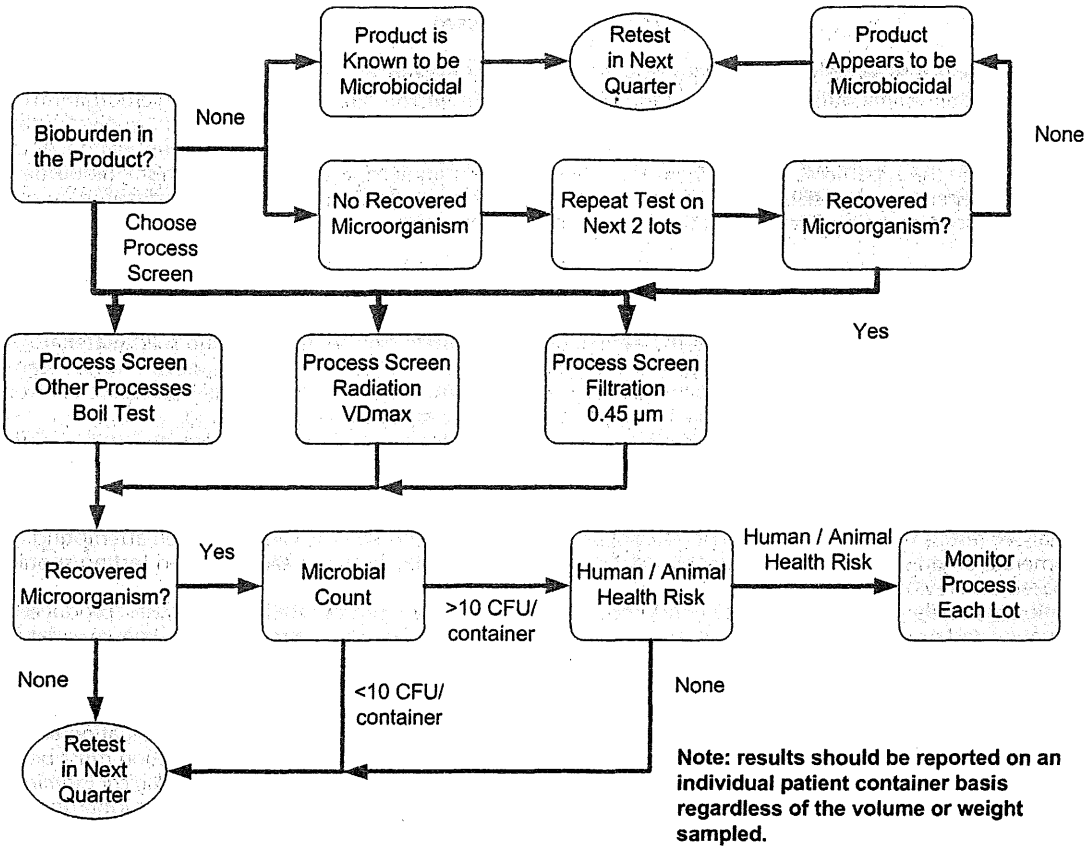

Fig. 1. Typical bioburden screening program.

Destructive Processes (except Radiation)

Microorganisms can be recovered after exposure to heat screening or shock at 100°, which eliminates vegetative cells and also triggers the germination process in spore-forming bacteria (*4*). Heat shock treatment is an effective means to reduce the amount of bioburden microorganisms to be evaluated because spore-formers are the most resistant to all lethal processes other than radiation. Heat screening eliminates vegetative cells that lack resistance to sterilization and encourages germination, thus making it easier to isolate spore formers.

Published reports compare the resistance of recovered microorganisms to that of predefined bioindicators for many common (*4,5*) sterilization processes. Analysts should evaluate the isolated spore-forming bacteria to determine their resistance D-value to specific lethal sterilization processes and thus to ensure the validity of the sterilization method. The available data describe the lack of resistance of medically and environmentally isolated bacteria, mold, and yeast species to the more commonly used sterilization methods, so analysts may find only limited value in repeating these resistance studies.

Radiation

Sterilization by radiation processes is based on the bioburden in the presterilized material. Analysts commonly use bioburden-based sterilization processes as described in AAMI/ISO 11137 (*6*). To ensure consistent sterility assurance, lot-to-lot variability of bioburden must be controlled. Data should be collected and analyzed for raw materials, intermediates, and/or products to ensure process control. AAMI/ISO 11137 provides detailed information about radiation sterilization practices, including cycle development, validation approaches, and dose-auditing methodologies including expectations for initial and periodic bioburden assessment. AAMI/ISO 11737 provides guidance for establishing methods to estimate bioburden levels on medical devices prior to irradiation (*7*).

Sterilizing Filtration

Bioburden screening for sterilizing filtration processes not yet subjected to formal validation can be performed by passing the material through a 0.45-µm-rated filter and examining the filtrate for viable microorganisms. Only those microorganisms that pass the 0.45-µm filter are of interest because they present the greatest potential challenge to the sterilizing filtration process. They should be evaluated against the upstream bioburden. Bioburden of greatest concern includes *Pseudomonas*, *Brevundimonas*, *Ralstonia*, and *Mycoplasma*.

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Identification

Recovered microorganisms can be identified to an appropriate level using the methods described in *Microbial Identification, Characterization, and Strain Typing* ⟨1113⟩. Only those microorganisms that present a potential risk to the product or the patient require identification to the species level or beyond. For a detailed discussion of the methods used to grow and identify microorganisms, see ⟨1113⟩. It is not necessary for the purposes of evaluating presterilization bioburden to identify all isolates to the species level, although this can be helpful in some investigations. Analysts must conduct all evaluative work with pure cultures, and they must apply normal microbiology laboratory procedures for the selection and maintenance of pure cultures.

Bioburden Control

A typical bioburden-control program includes review and analysis of potential sources of contamination as well as sound process design and preventive and monitoring measures. The microbiological contamination-control program should be developed to identify and control bioburden and to assess product risk based on a formal assessment of risk modalities. The bioburden risk assessment should result in the establishment of critical control points and should include consideration of the following elements:

- Microbiological attributes of materials before sterilization and the manufacturing process used for the materials (if applicable)
- Inherent antimicrobial properties of the materials
- Time limits for process execution
- Water activity of the material
- Environmental conditions within the facility
- Equipment design and cleaning
- Sanitization, decontamination, and other active microbial control processes (such as prefiltration, temperature, pH, osmolarity, etc.)

Controlling the bioburden of materials and products to be sterilized will ensure conformance to the levels required by the sterilization process validation. Additionally, controlling the bioburden levels of the items to be sterilized assures that residuals (e.g., allergens, endotoxins, and exotoxins) from that population will also be controlled. This is important because direct detection of these materials is challenging.

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⟨1229.4⟩ STERILIZING FILTRATION OF LIQUIDS

INTRODUCTION

Sterilization processes are divided broadly into two categories: destruction of microorganisms and their physical removal from the material to be sterilized. Autoclaving is an example of the former, and sterilizing filtration is an example of the latter. The physical removal of microorganisms depends on the bioburden of the solution to be filtered, the properties of the solution, the filtration conditions, and the filter itself.

Sterilizing filtration is a process that can be validated to consistently yield filtrates that are sterile, as defined in *Sterilization of Compendial Articles* ⟨1229⟩. This chapter provides an overview of (1) various factors that affect the filtration process, (2) the filter integrity test and when to perform it, (3) prefiltration bioburden control, (4) responsibilities of the filter manufacturer and user, and (5) troubleshooting the filtration process.

Multiple factors contribute to the effectiveness of any sterilizing filtration process. These include the type and number of microorganisms, the properties of the liquid, the filter design and membrane polymer, and the filtration process parameters. Properties of the liquid that influence filtration effectiveness include its chemistry, viscosity, surface tension, pH, osmolarity, ionic strength, and temperature, as well as the presence of insoluble materials. Aspects of the filter that affect the filtration include effective filter area, nominal pore size, pore-size distribution, membrane thickness, porosity, membrane polymer, filter pleat density, nonwoven support layers, electrostatic charge, and the hydrophilic nature of the filter membrane. The filtration

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process parameters that influence microbial retention include temperature, flow rate, volume, filtration time, differential pressure, and pressure pulsations.

Additionally, effective sterilizing filtration depends on (1) the production controls and quality assurance systems used by the filter manufacturer to ensure the quality and uniformity of the filter membranes and fabricated filters, (2) the qualification and validation studies conducted by, or for, the filter user to demonstrate that the chosen sterilizing filtration process achieves a sterile filtrate, (3) effective controls to ensure that prefiltration bioburden and operating parameters remain within the validated ranges, and (4) filter integrity. Filter users should ensure that the filtrate remains sterile by using validated sterilization processes for the filtration assembly and all downstream manufacturing equipment and effective aseptic handling of the sterilized materials. Filter users should carefully consider placement of the filter (e.g., proximity to the filling line or hold tank) to minimize the possibility of postfiltration contamination.

Sterilizing-Grade Filters

A sterilizing-grade filter is one that is capable of retaining a minimum 1×10^7 cfu of *B. diminuta* (ATCC 19146) per square centimeter of effective filter area when tested in accordance with ASTM F838-05 (2013), *Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration (2)*.

The designation "sterilizing grade" implies a sterilizing action only if other conditions are met, including the integrity test specification established by the filter manufacturer and validated by the user (see *Validation*, below).

Sterilizing-grade filters typically are microporous membranes that have nominal pore-size ratings of about 0.2 µm. These membranes are fabricated with various materials, have relatively narrow pore-size distributions, and can be integrity tested. The integrity test results can be correlated with microbial retention. Membrane filters that have a nominal pore size of 0.45 µm can be validated to produce sterile filtrates under some conditions; for example, some liquids require high differential pressures to achieve useful flow rates, and these pressures are not suitable for use with 0.2-µm–rated filters. When manufacturers use 0.45-µm filters, they should ensure particularly stringent control of presterilization bioburden.

Retention Mechanisms

Microporous membranes remove microorganisms from the liquid by two primary mechanisms: sieve retention, which relies on physical blockage of particles that are larger than the pores they encounter, and adsorption, which is a charge-related phenomenon whereby particles are bound to the membrane surfaces. Both mechanisms should be considered during the development, qualification, and validation of sterilizing filtration processes.

Sieve retention, for the most part, is independent of filtration conditions and the microbial challenge level, but the composition of the liquid, temperature, and filtration conditions can affect sieve retention under certain conditions.

Adsorption is a charge-related phenomenon that is influenced by the composition of the membrane, the properties of the filtered liquid, the filtration conditions, and the number and type of microorganisms present in the liquid.

The number and type of microorganisms and other particles present in the material to be filtered can affect retention. Sieve retention implies that every microorganism is larger than the largest pore in the filter. Microorganisms and filter pores are not uniform in size, and some microorganisms may be smaller than the largest pores. Even if a microorganism is smaller than the pore it encounters, it may be retained if a site is available and the filtration conditions are conducive to adsorption. Retention probability is related to the number of microorganisms in the upstream bioburden *(4,5)*.

FACTORS THAT AFFECT RETENTION

Nature of Pores

Microporous membranes consist of a polymer matrix that is penetrated by interstices commonly referred to as pores. Compared with those in depth filters, the pores in microporous membranes have a relatively narrow size distribution. The size, number, and shape of the pores determine the filter's retention capabilities. With the exception of the pores in track-etched filter membranes, pores are not cylindrical; they are made up of a series of pseudopolyhedral structures with varying internal diameters *(6)*.

The pore size, pore-size distribution, and membrane porosity are a function of the manufacturing process. Careful design and control of that process are necessary to ensure that the resulting membrane has the desired integrity test value, microbial retention capability, and uniformity.

Nature of Microorganisms

Microorganisms have a variety of shapes and sizes. If the microorganism encounters a membrane pore that is smaller than its smallest diameter, the microorganism likely will be captured by sieve retention. If, however, the microorganism is smaller than the pore it encounters, it may be retained by adsorption if the residence time, electrostatic charge, pH, fluid chemistry, and membrane material are conducive to adsorption.

Some genera of microorganisms are deformable, so that at high-pressure differentials or flow rates a microorganism may be forced through a filter pore that is only slightly smaller than the organism *(7)*. Mollicute bacteria lack cell walls and thus are small and pliable enough to pass through filter membranes under certain conditions.

Grow-through (i.e., passage of microorganisms through a filter as a function of time) may result from one or more scenarios. The microorganism may penetrate as it multiplies: a parent cell divides into two smaller daughter cells that negotiate pore passageways. Penetration can occur with time, because the increasing number of microorganisms overwhelms the few larger pores that are encountered. However, because of the limited time periods typically involved, the controls on bioburden, and

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the often limited availability of nutrients, grow-through is considered a rare phenomenon in pharmaceutical processes (see *Monitoring of Bioburden* ⟨1229.3⟩) *(8)*.

Composition and Structure of the Filter Matrix

Several factors related to the filter matrix can affect microbial retention. These include the material from which the filter is made, the pore size and pore-size distribution, whether the membrane is isotropic or anisotropic (i.e., whether membrane pore structure is uniform from face to face or "tapers" from one face to the other), membrane thickness, and whether the filter consists of single or multiple layers.

The membrane material is especially important if adsorption is a significant mechanism in a particular filtration scheme. For example, polyamide exhibits stronger microbial adsorption than does cellulose ester *(3)*.

Microporous membranes that have a relatively wide pore-size distribution are less likely to retain microorganisms, particularly at high challenge levels, than membranes of comparable pore-size ratings with narrower pore-size distributions. This relates to the probability of a microorganism's encountering a pore larger than itself.

Thicker membranes generally are more retentive than thinner membranes of the same type and pore-size rating, owing to the higher probability of entrapment or adsorption within the pore structure because of the increased distance that bacteria must travel in thicker membranes. This distance favors entrapment, as does increased residence time within the pore, which favors adsorptive retention *(3)*.

Multi-layered membrane filters exhibit a higher probability of retention than do single-layered membrane filters of the same thickness because the small number of largest pores is the factor affecting retention and the probability of a large pore in one layer being congruent to a large pore in the adjoining layer of a double-layer filter is negligible *(3)*.

Composition of the Filtered Solution

The composition of the filtered solution can adversely affect the membrane material if an incompatibility exists, causing damage to the membrane and affecting both retention and physical integrity, unless detected before the filter is selected for use. In addition, if adsorption is a significant retention mechanism, then solution properties such as pH and the presence of surfactants become important.

Surface charge and ionic strength are important variables. Bacterial and membrane surfaces in aqueous media are negatively charged, resulting in repulsion. The repulsive force is balanced by attractive forces, which include hydrophobic surface energy minimizing forces, operative only over short distances, and hydrogen bonding. High ionic strength allows the surfaces to close —because of discharge through the electrolyte—to the point where hydrophobic adsorption can occur *(3)*. Also, high ionic strength can draw water out of the cell, reducing its size, which may lower the probability of retention, depending upon the composition of the prefiltration bioburden. Surface tension and the presence of surfactants influence retention. Adsorption of surfactant by the filter and the microorganism creates repulsion, leading to a decreased probability of retention. Surfactants in concentrations as low as 0.05% have been shown to inhibit adsorption and decrease the retention of latex spheres *(9)*.

Filtration Conditions

Differential pressure, flow rate, and temperature are among the factors that can affect microbial retention. Hydraulic shock should be avoided, not only because it affects pressure differential and flow rate but also because it can damage the filter. Flow rate is proportional to differential pressure, and higher flow rates reduce adsorption because the contact time is reduced *(10)*.

Microbial retention may be reduced at higher temperatures when these result in higher flow rates due to decreased solution viscosity. Temperature effects are not significant in filters where sieve retention is the primary removal mechanism.

Filter Efficacy: Log Reduction Value

Filtration efficacy can be defined in terms of a log reduction value, which is the logarithm of the quotient produced by dividing the upstream challenge population by the recovered downstream population.

The log reduction value is influenced by the number and size of the challenge microorganisms, the filter design and membrane polymer, the filtration process parameters, and the properties of the solution.

To determine the specific log reduction value of a filter, the challenge test should permit some passage of the test microorganism through the filter in order to produce a denominator. Sterilizing-grade membrane filters should not permit passage of the specified challenge microorganisms for that filter rating. For this reason the log reduction value of sterilizing-grade filters is described as equal to or greater than the log of the challenge population.

Validation

As noted, microbial retention in sterilizing filtration relies on a combination of sieve retention and adsorption. Validation of sterilizing filtration therefore requires determination of the effect of the liquid on the filter, determination of the effect of the filter on the liquid, and demonstration that the filter can consistently yield sterile solutions under the intended conditions of use. The liquid to be filtered can affect the pore structure of the membrane, can have electrostatic properties different from the standard challenge suspension used to establish integrity test specifications, and can change the size and shape of the challenge microorganisms. Factors that should be considered when developing a sterilizing-filtration validation protocol include the surface tension, pH, temperature, and osmolarity of the liquid to be filtered; the compatibility of the material or solution components with the filter itself; the pressures, flow rates, and hydraulic shock likely to be encountered; and the maximum

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filtration time and volume to be filtered. The effect of sterilization (steam, radiation, or gas) on the filter's retention capability also should be considered.

B. diminuta (ATCC 19146) is used as the challenge organism unless it is not viable in the liquid to be filtered. Viability studies should be used to confirm that the liquid has no adverse effects on the challenge organism. If the challenge organism is viable in the liquid to be filtered, the liquid should be inoculated to achieve a challenge level of 1×10^7 cfu/cm^2, and the filtrate should be evaluated for the presence of the challenge organism. If *B. diminuta* is not viable in the liquid, several options are available, and analysts can (1) modify the liquid to ensure the viability of the challenge organism (e.g., adjust pH or remove the bactericidal component), (2) reduce the exposure time to ensure that the challenge organism remains viable, or (3) change the challenge organism from *B. diminuta* to one that has been isolated from the liquid to be filtered. These studies should employ production process pressure differentials or process flux values as appropriate.

If possible, the liquid to be filtered should be used because in some instances the challenge organism has penetrated a filter in contact with the liquid but has been retained by the same filter when inoculated into a surrogate fluid *(11)*.

Three different lots of filter membranes should be used for the microbial retention studies. The membranes should have preuse integrity test values that are near the filter manufacturer's specification in order to minimize the possibility that production filters will fail to meet the integrity test value established during the validation exercise. Successful microbial challenge studies result in no microorganisms detectable in the filtrate. The sterilization process for the filter, its housing, and associated equipment should be validated. The filter should be sterilized within its housing rather than relying on aseptic assembly following sterilization of the filtration system components. The assembled filtration apparatus can be steam sterilized in an autoclave, using a vacuum cycle, with particular attention to the orientation and wrapping of the housing and any associated tubing to allow condensate drainage and steam penetration. Validated steam sterilization-in-place cycles also can be used.

Sterilization methods and cycles should be carefully chosen and designed to preclude damage to the filter. Pre- and poststerilization integrity testing can be used to confirm that the sterilization procedure does not change the integrity test value and to demonstrate that the sterilization process does not damage the filter.

Finally, the scale of the validation approach should be considered. Two approaches are common. One uses a small section of membrane material, typically a 47-mm disk, to represent the filter. This approach validates the microbial retention capability of the membrane. A second approach uses the intended filter configuration, typically a 10-inch cartridge in its housing. This approach validates the filter system performance in addition to the retention capability of the membrane material. Analysts should consider the volume of the test liquid and operational considerations when they choose which approach to use.

Integrity Testing Principles and Methods

Integrity testing can be used to show that a filter has the correct pore-size rating, is installed properly in its housing, and has not been damaged by the process used to sterilize it. These integrity test methods generally rely on detecting gas flow caused by pressure differential across a wetted membrane. *Figure 1* shows the relationship between gas flow and pressure differential for high-surface-area and low-surface-area membranes (a 47-mm membrane disk can be considered low surface area, and a multicartridge array of 10-inch cartridge filters can be considered high surface area).

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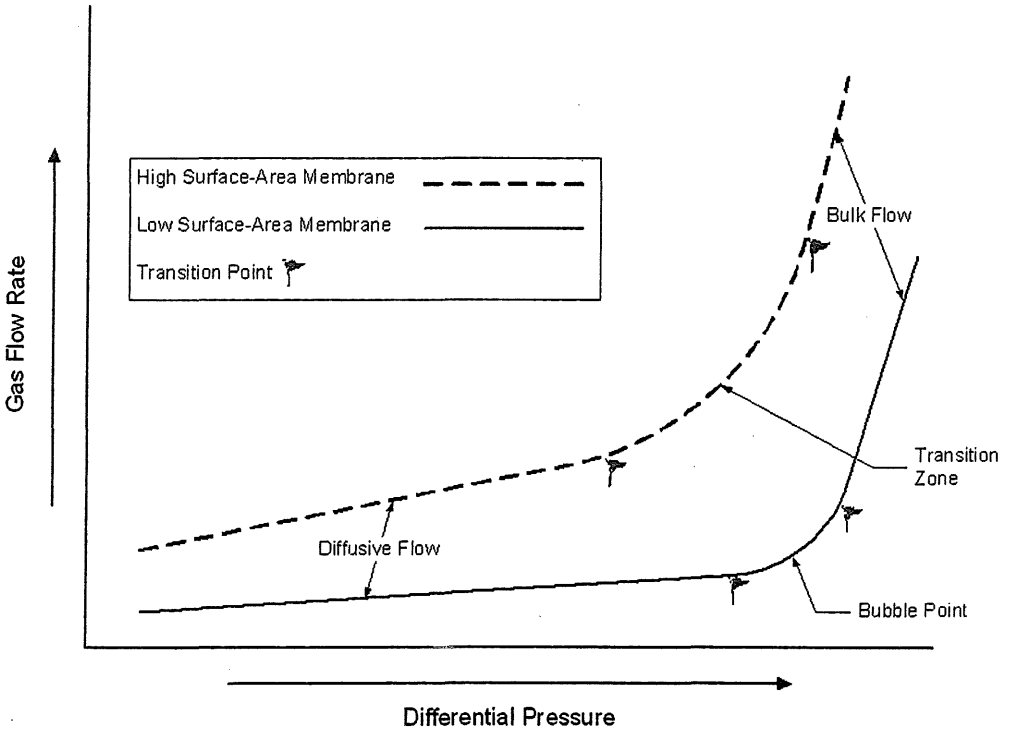

Figure 1. Relationship between gas flow and pressure differential for high-surface-area and low-surface-area membranes (see the section *Integrity Testing Principles and Methods* for definitions).

A filter that successfully passes an integrity test based on specifications developed during an effective sterilizing filtration validation study is capable of producing a sterile filtrate.

Integrity test methods for microporous membrane filters include bubble point, diffusive flow, and pressure hold. Membrane type and size and the particular filtration process influence the choice of an appropriate integrity test method. The sensitivity of the bubble point test decreases with increasing filter area because of increasing diffusive flow. Bubble point, diffusive flow, and pressure-hold integrity tests are used for hydrophilic microporous membrane filters. As shown in *Figure 1*, diffusive flow occurs at pressures below the bubble point, and bulk flow takes place above the bubble point. The bubble point marks the transition between diffusive flow and bulk flow. The exact bubble point is difficult to detect in high-surface-area filters, because the high membrane surface area generates significant diffusion. Integrity testing may be performed manually or by automated equipment designed specifically for that purpose.

BUBBLE POINT

The bubble point occurs when a gas displaces a wetting liquid from the largest membrane pores, resulting in bulk gas flow through those pores. The flow is evidenced by a steady stream of bubbles through a column of water on the downstream side of the membrane.

The bubble point relates to the pore size of the membrane and the contact angle that the wetting liquid makes with the pore wall. Bubble point is indirectly proportional to membrane pore size and is directly proportional to the surface tension of the wetting liquid; that is, filters with smaller pores have higher bubble points than those with larger pores, and liquids that easily wet the membrane exhibit higher bubble points than those that do not. Bubble point is defined as:

$$P = 4 \times \gamma \times \cos\Theta / D$$

P = pressure to evacuate the pore
γ = surface tension
Θ = angle of wetting
D = pore diameter

Microbial retention and bubble point correlate: numerous studies have demonstrated that when the microbial reduction ratio is plotted against the bubble point, a line of constant slope results *(12)*.

The actual bubble point is independent of the membrane surface area, but diffusive flow through high-surface-area filters can mask the true bubble point. The bubble point test is easy to perform on small- to medium-scale filters, the test time is short, and temperature effects are minor. Manual bubble point testing requires manipulation of the downstream side of the filter and is subjective.

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DIFFUSIVE FLOW

In diffusive flow testing a wetted filter provides a liquid layer across which gas can flow by means of diffusion. Diffusive flow is measured directly at constant pressure.

Diffusive flow is proportional to the differential pressure of the test gas, the diffusivity of the test gas in the wetting liquid, the thickness (depth) of the wetting liquid, the porosity (i.e., void volume) of the membrane, and the effective filtration area. Diffusive flow is defined by Fick's Law of Diffusion, shown as:

$$N = [D \times H \times (p_1 - p_2)]/(L \times \rho)$$

N = permeation rate (moles of gas per unit time)
D = diffusivity of the gas in the liquid
H = solubility coefficient of the gas
$p_1 - p_2$ = transmembrane pressure (differential pressure)
L = thickness of liquid in the membrane
ρ = void volume (porosity) of the membrane

Unlike the bubble point test, diffusive flow testing measures gas flow through all the wetted pores, and thus diffusive flow does not correlate directly with microbial retention. However, bacterial challenge tests with a series of filters that have decreasing diffusion rates show that a gas diffusion rate exists below which sterile filtrates are obtained.

Diffusive flow testing is highly sensitive, especially for higher-surface-area membranes. Larger pores or flaws can be detected by a thinning of the liquid layer and correspondingly higher diffusive flow rates. Diffusive flow testing is useful for membranes with small pore sizes (e.g., 0.1 µm and smaller) because of the high pressures required for bubble point testing. Diffusive flow testing measures flow across the total pore volume, which may mask a flaw, especially in high-surface-area multiple-cartridge arrays *(13)*. This test also is highly sensitive to temperature.

PRESSURE HOLD

The pressure hold integrity test is a variation of the diffusive flow test. The wetted membrane provides a liquid layer across which gas can flow by means of diffusion. The gas flow is proportional to differential pressure and is measured by pressure decay on the upstream side of the membrane.

The rate of pressure decay is influenced by upstream volume of the particular holder–filter combination, valve placement, and tubing volume. Temperature should remain constant because of the relationship between temperature and pressure defined by the ideal gas law.

Conversion of pressure decay test results to diffusive flow values allows correlation with microbial retention. Analysts establish the relationship between pressure decay and diffusive flow by calculating diffusive flow on the basis of pressure drop per unit time, with a known upstream volume and reference pressure. Diffusive flow as it relates to pressure decay is shown as

$$D = [(p_1 \times V_1)/(p_0 \times t)] \times ln[p_1/(p_2 - \Delta p)]$$

D = diffusion
p_1 = starting test pressure
V_1 = upstream volume of filter system
p_0 = atmospheric pressure
t = test time
p_2 = ending test pressure
$\Delta p = p_1 - p_2$

The advantages and disadvantages of the pressure-hold test are similar to those of the diffusive flow test. The pressure-hold test has the additional abilities of revealing imperfections in the assembly and sealing of the housing and filter seating and avoiding downstream manipulation. Its disadvantages are that it is strongly influenced by temperature and that accurate measurement of the upstream volume is required *(14)*.

When to Test Integrity

The decision not to perform preuse, presterilization integrity testing should be based on a formal risk assessment.

Preuse, poststerilization integrity testing may create an unnecessary risk for microbial contamination of the filter and associated downstream tubing and equipment. Preuse, poststerilization integrity testing is unnecessary if effective validation studies have demonstrated that the process for sterilizing the filter does not affect the integrity test value of the filter.

Postfiltration integrity testing should be conducted to ensure that the filter was not damaged during the filtration process.

PREFILTRATION BIOBURDEN CONTROL

The bioburden removal capability depends on the available filter retention capacity, which is a function of the inherent bioburden load present in the entire volume of the liquid to be filtered and the effective filter surface area. Studies have demonstrated that microbial retention in sterilizing filtration is a function of the upstream bioburden *(3,15,16)*. Also, nonviable particulate matter that may be present in the solution can influence the retentive capacity of the filter *(17)*. Therefore, the

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prefiltration bioburden and particulate levels of the solution should be minimized and controlled before the final sterilizing filtration step.

Various filter configurations and processes can be used to control the bioburden and nonviable particulate levels presented to the final, sterilizing-grade filter. One configuration is a multifilter arrangement that consists of two sterilizing-grade filters (or a bioburden reduction filter followed by a sterilizing-grade filter) connected in series (*Figure 2*).

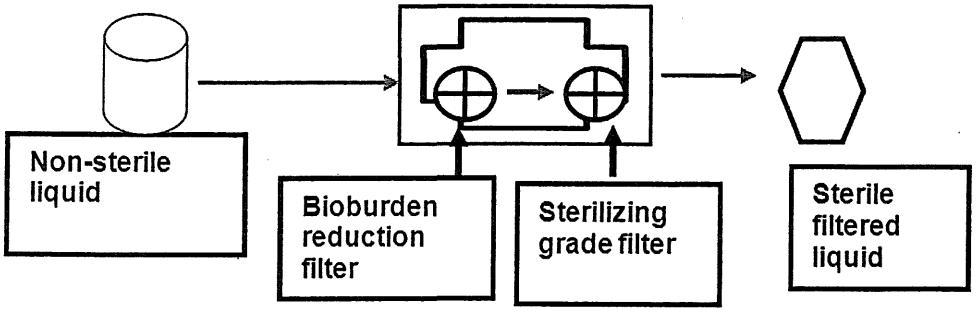

Figure 2. Multifilter configuration to control bioburden and nonviable particulates.

Another configuration appropriate for prefiltration bioburden control uses two filtration steps separated in time: the liquid is sterile-filtered into a sterilized tank, where it is then held before a final, sterilizing filtration (*Figure 3*).

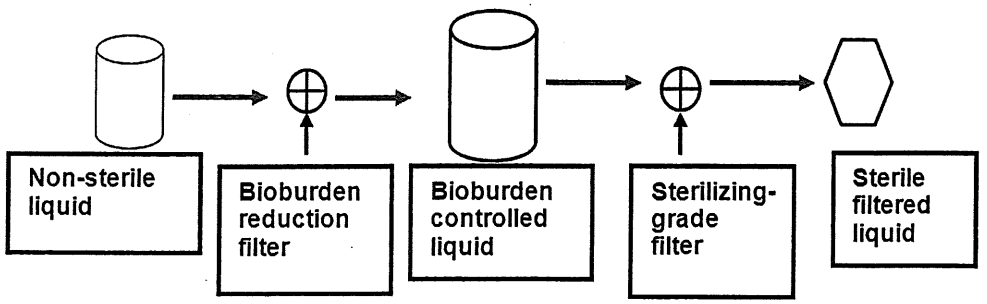

Figure 3. Bioburden control using two filtration steps.

In each of these scenarios, the bioburden and particulate levels presented to the final, sterilizing-grade filter are low and are controlled by prefiltration.

When the process results in a consistently low and controlled prefiltration bioburden, use of a single sterilizing-grade filter is appropriate.

Irrespective of the strategy employed, validation studies should demonstrate the capability to consistently achieve the requisite levels of prefiltration bioburden and particulate level reduction and control.

RESPONSIBILITIES

Filter Manufacturer

The filter manufacturer is responsible for ensuring that the filter production process has been validated and is well controlled and that the sterilizing-grade filters meet the requirements of ASTM F838-05 (2013). The filter manufacturer determines the integrity test specification for the filters, usually adding a safety factor to ensure that each filter will meet that specification. The filter manufacturer conducts extractable and leachable studies to ensure that the filter does not release objectionable levels of these materials into the solvent systems typically employed in pharmaceutical manufacturing. The filter manufacturer conducts cleanliness tests to assure that the filter does not adversely affect the USP particulate requirements of the product. The filter manufacturer provides technical support and troubleshooting advice if the filter user encounters a problem.

Filter User

The filter user is responsible for establishing microbial retention at the validated integrity test value, establishing microbial retention in the liquid to be filtered, and validating the use and sterilization of the filter and housing. The filter user is responsible for determining that the filter is not additive or extractive to the extent that the filtered liquid is adversely affected.

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TROUBLESHOOTING

Failure of a filter to pass an integrity test may mean that the filter is damaged, is improperly sealed in the housing, is incompletely wetted, or is nonintegral. It also could mean that the filter is incorrectly labeled (e.g., has the wrong pore size) or that the integrity test apparatus has been improperly set up or calibrated.

The cause of an integrity test failure can be determined by evaluating the test setup, test parameters, wetting fluid, and wetting procedure; ensuring that the system is leak-free and the temperature is constant; and ensuring that the test equipment has been properly calibrated.

If the cause of the failure cannot be determined, analysts can rewet the filter and repeat the integrity test, increasing flush time, flush volume, and pressure differential. It may be beneficial to use a lower surface tension reference fluid (e.g., 70% isopropyl alcohol). If the filter fails the integrity test using the reference fluid, it should be considered nonintegral. In addition, the filter can be returned to the manufacturer for a full analysis to further elucidate the cause of the integrity test failure.

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⟨1229.5⟩ BIOLOGICAL INDICATORS FOR STERILIZATION

INTRODUCTION

A biological indicator (BI) is a well-characterized preparation of a specific microorganism that has known resistance to a specific sterilization process.[1] BIs are used to demonstrate the effectiveness of processes that render a product sterile in its final package or container, as well as the effectiveness of the sterilization of equipment, product contact materials, and packaging components as required. BIs may also be used to monitor established sterilization cycles and are used for periodic reassessment of sterilization process effectiveness. BIs are process aids and can support the correlation of physical parameters to microbiological destruction. Microorganisms recognized as suitable for BIs are spore-forming bacteria, because the spores of these microorganisms are significantly more resistant than the vegetative cells that comprise the majority of bioburden in or on materials.

[1] Microbial retention challenges as described in *Sterilizing Filtration of Liquids* ⟨1229.4⟩ are not BIs.

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PROPER USE OF BIOLOGICAL INDICATORS

BIs provide microbiological evidence of process effectiveness that should be correlated to physical measurements (see *Sterilization of Compendial Articles* ⟨1229⟩). Microbiological resistance to sterilization varies with the physical conditions; however, there are no established means for accurately predicting microbial destruction based solely on physical measurements, with the exception of radiation sterilization. BI placement locations within or on materials to be sterilized are chosen to confirm that the desired sterilizing conditions have been attained.

BIs are typically spore-forming bacteria of the genera *Geobacillus*, *Bacillus*, and *Clostridium*. Preference in BI selection should be given to well-characterized strains.

RESPONSIBILITIES

BI Manufacturer's Responsibility

The responsibility for determining the performance characteristics of each BI lot resides with the BI manufacturer. The manufacturer should provide, with each lot of BIs, a certificate of analysis that attests to the validity of BI performance claims. The manufacturer should provide information concerning the microbial population and resistance (*D* and *z* values, respectively, where appropriate) as well as storage and expiry information. The BI manufacturer may choose to include survival or kill times for the BI in their documentation. The resistance of the BI should be determined by the manufacturer under defined conditions. The manufacturer should provide directions for use, including the medium and conditions used for the recovery of microorganisms after exposure to the sterilization process. Disposal instructions also should be provided by the manufacturer of the BI.

BI User's Responsibility

When BIs are purchased, their suitability for use in a specific sterilization process must be established. The BI user should obtain a certificate of analysis for each lot of BIs and verify the manufacturer's label claims for spore population (see *Biological Indicators—Resistance Performance Tests* ⟨55⟩). When a BI is used in accordance with the BI manufacturer's directions, the resistance of the BI need not be reconfirmed.

User-Prepared Biological Indicators

A user of BIs may elect to propagate spore crops of a single species for use as a suspension. Alternatively, these spore suspensions may be purchased from a BI manufacturer. When liquid suspensions are applied to a substrate, it is the user's responsibility to determine the population and resistance of the microorganism used. The resistance determined for liquid suspensions relates only to other lots of the same suspension and is not representative of how that microorganism will perform on a substrate or in a different suspending medium. In these circumstances, the BI resistance and population should be re-established (see ⟨55⟩).

CHARACTERIZATION OF BIOLOGICAL INDICATORS

The use of BIs should include procedures for their acceptance and control. The following elements outline the major considerations. Resistance performance is addressed separately in ⟨55⟩, which provides methods for evaluating BI resistance.

Packaging and Storage

Store under the conditions recommended on the label or under validated conditions, and protect from light, toxic substances, excessive heat, and moisture.

Expiration Date

Use within the BI's labeled or determined expiration date.

Identification

Where identification of the BI species is deemed necessary, as in the course of an investigation into unusual results, use either a phenotypic or genotypic identification method (see *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩ for additional information).

Purity

By examination of the colonies derived from the spores on a suitable plate culture medium, determine that there is no evidence of contamination with other microorganisms.

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Disposal

Prior to discarding used spores, sterilize using a method recommended by the BI manufacturer or other equivalent means.

TYPES OF BIOLOGICAL INDICATORS

A BI is a well-characterized preparation of a specific bacterial spore of known resistance to a specific sterilization process. Some BIs may contain two different species and concentrations of bacterial spores for use in the evaluation of two different sterilization processes.

One form of BI preparation includes spores that are placed on a carrier (e.g., a disk or strip of paper, glass, plastic, metal, or other material) and may be packaged to maintain the integrity and viability of the spores inoculated onto the carrier. The carrier and primary packaging should not be damaged or degraded by the specific sterilization process. Another preparation of BIs is a spore suspension that is inoculated on or into representative units of the article to be sterilized. A surrogate article may be used if it is not practical to inoculate the actual article. A surrogate article is a preparation that differs in one or more ways from the actual article but performs as the actual article during cycle development, validation, and routine use. The physical design of actual or surrogate articles can affect the resistance of spore suspensions that are inoculated on or into an article (see ⟨55⟩). In the case of liquid inoculated products, it is essential to determine the population, *D* value (and, in terminal sterilization applications, *z* value) of the relevant BI spore in the liquid product, and any simulated product substrate (if utilized).

A third form of BI is a sealed system that includes the growth medium (either in direct contact with the BI during the sterilization or placed in contact with the BI after sterilization) for recovery of process-exposed BI microorganisms. Some BI systems may contain a growth indicator or sensor in addition to growth media.

SELECTION FOR SPECIFIC STERILIZATION PROCESSES

The selection of a BI requires knowledge of the resistance of the BI system to the specific sterilization process. It must be established that the BI system provides a challenge to the sterilization process greater than the resistance of the native bioburden. The recommendations for BI with each sterilization process are not exclusive; they represent only the more common choices.

Steam Sterilization by Direct Contact

For steam sterilization by direct contact, the commonly used BI contains spores of *G. stearothermophilus* (ATCC 12980 or ATCC 7953), a thermophilic microorganism with a moist heat resistance substantially greater than that of most vegetative microorganisms (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩).

Moist Heat Sterilization of Aqueous Liquids

Heat-resistant spore-forming microorganisms such as *C. sporogenes* (ATCC 7955), *B. subtilis* (ATCC 35021), or *B. atrophaeus* (ATCC 9372) are used. *B. subtilis*, *B. atrophaeus*, and *C. sporogenes* are preferred for use in sterilization of aqueous solutions or where their lower thermal resistance is more appropriate.

Dry Heat Sterilization

For dry heat sterilization, spores of *B. atrophaeus* (ATCC 9372) are typically used (see *Dry Heat Sterilization* ⟨1229.8⟩). Where dry heat depyrogenation has been demonstrated (*Dry Heat Depyrogenation* ⟨1228.1⟩), sterilization by dry heat need not be confirmed, and a BI is not required. The elevated temperatures required to depyrogenate materials are more than sufficient to sterilize the materials at the same time.

Ionizing Radiation

The use of a resistant BI is unnecessary for the evaluation of radiation sterilization processes. Dose setting involves the evaluation of preirradiation bioburden as well as dosimetric evaluation and allied tests as defined in ISO 11137-1, -2, and -3, as well as in *Radiation Sterilization* ⟨1229.10⟩.

Gas Sterilization

For ethylene oxide sterilization, spores of *B. atrophaeus* are commonly used. For other gaseous agents, spores of *G. stearothermophilus* or *B. atrophaeus* are commonly used (see *Gaseous Sterilization* ⟨1229.7⟩).

Chemical Sterilization

The sterilization of items using a liquid sterilant is accomplished using spores of an appropriate strain such as *B. atrophaeus*, *B. subtilis*, or other appropriate spore-forming species, as determined by the user. Whichever strain is chosen for this purpose should have greater resistance than does the bioburden.

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Vapor Phase Sterilization

The biphasic nature of these materials precludes the accurate determination of specific lethal conditions (for establishment of *D* values, see *Vapor Phase Sterilization* ⟨1229.11⟩). BIs using either *G. stearothermophilus* or *B. atrophaeus* have been utilized in the evaluation of these processes.

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⟨1229.6⟩ LIQUID-PHASE STERILIZATION

INTRODUCTION

Microorganisms are subject to destruction in a variety of ways. Aside from the classical methods of steam, dry heat, and radiation, destructive sterilization may also occur by immersion in a chemical solution. This is termed liquid-phase sterilization *(1)*. A number of chemical agents, such as aldehydes, acids, bases, and strong oxidants in solution, under the appropriate conditions, are capable of destroying bacteria and fungi, including both vegetative cells and spores in a quantitative fashion *(2,3)*. Objects to be sterilized are immersed in the solution of the chemical agent, after which the agent must be removed in a manner that preserves the sterilized object from recontamination. Removal of the chemical sterilant from the exposed surfaces that have been sterilized must be accomplished in a manner that maintains the sterility of the item postprocessing. Recontamination falls outside the scope of usual consideration for sterilization processes. However, in liquid chemical sterilization it is customary to include the agent's removal (whether this is accomplished by physical or chemical means) in the overall process, together with any needed additional steps to avoid recontamination.

A substantial number of liquids in aqueous solution are capable of sterilizing articles during immersion. Examples include the following:

- Aldehydes—glutaraldehyde, formaldehyde
- Acids—peracetic acid, nitric acid, sulfuric acid
- Bases—sodium hydroxide, potassium hydroxide
- Oxygenating compounds—hydrogen peroxide, ozone, chlorine dioxide
- Halides—sodium hypochlorite, chlorine

As is the case for gas sterilization, the effectiveness of chemical sterilants varies with concentration and temperature. Other factors that affect antimicrobial activity include pH, extent of mixing (if present), and presence of cellular or other debris. Because of the limited number of variables, process control for sterilization by liquids is relatively simple.

Because there are no widely accepted biological indicators for sterilization by liquids, the use of a common mesophilic sporeformer such as *Bacillus atrophaeus* or *B. subtilis* is commonplace because these are the likely worst-case bioburden isolates.

The agents used for sterilization by liquids vary with respect to sterilant stability, effective pH range, concentration, temperature, contact time required, and potential interaction with the materials. When selecting the most appropriate sterilant, manufacturers must consider its effect on the materials, package components, and equipment, as is the case with all other sterilizing processes. The variety of agents, process diversity, and potential applications preclude a material-by-material review of these agents in this chapter. Manufacturers should note that these agents are highly toxic, and appropriate safety measures should be practiced at all times during cycle development, validation, and routine operation.

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VALIDATION OF STERILIZATION BY LIQUIDS

Experimental evidence has shown that first-order kinetics is appropriate for microbial destruction, which makes validation a simple exercise. The validation of sterilization by liquids can be accomplished using either the half-cycle approach or the bracketing method.

Half-Cycle Approach

The half-cycle approach described below is a modification of the method described in *Gaseous Sterilization* ⟨1229.7⟩ (see *Figure 1*). It is a method that requires the destruction of a resistant microorganism at defined lethal conditions. In routine operation, the process dwell period is arbitrarily doubled and supports a theoretical reduction of the biological indicator (and thus the bioburden) to a probability of a nonsterile unit of at least 10^{-6} for a full cycle.

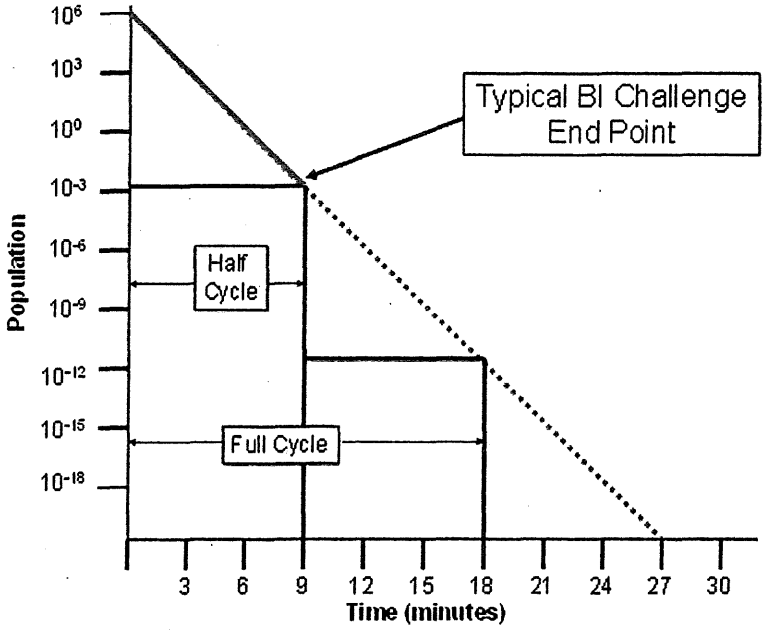

Figure 1. Half-cycle sterilization validation.

The half-cycle method originally was used for ethylene oxide sterilization when the relationship between the microorganisms and the delivered process parameters was less certain.

Bracketing Approach

In this method (see *Figure 2*), analysts evaluate conditions of concentration and temperature that bracket the defined process condition to support both over- and under-treatment of the materials and bioburden, respectively. Users can establish the death rate for the microbial population for each of the conditions that bracket the routine process.

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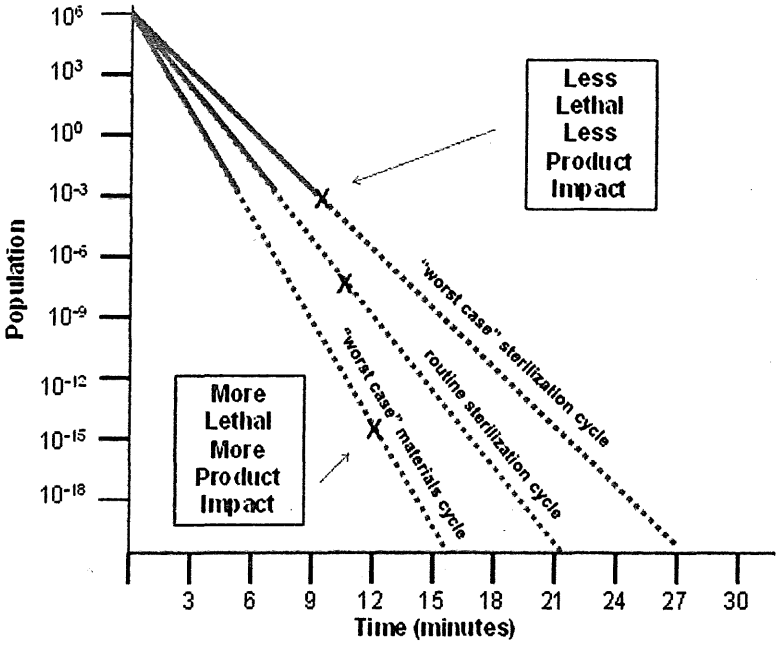

Figure 2. Bracketing method.

Regardless of the approach used, in order to complete the cycle development and validation manufacturers must identify a rapid neutralization method that inactivates the chemical agent to allow microbial quantification after fractional kill exposure. The exposure periods may need to be brief because many of these agents have rapid kill rates.

Equipment Qualification

Equipment qualification is a predefined program that examines the equipment to confirm that it has been properly installed and operates as intended before the sterilization process. This activity for sterilization by liquid chemicals is simple, because the equipment used is rarely complex. Temperature control and agitation/recirculation rates are the essential considerations.

Component and Load Definition

Sterilization by liquids is a surface phenomenon, and all surfaces of the materials must be immersed in the sterilant. Treatment uniformity can be ensured by recirculation or mixing of the sterilant during the process. Penetration into needle lumens, closely fitted parts, and porous materials should be confirmed. The use of a maximum load per defined vessel or container represents the worst case because it provides the maximum surface area to be sterilized.

Biological Indicators

The common indicator organisms for chemical sterilization are *B. atrophaeus* ATCC 9372 or *B. subtilis* ATCC 6633. The spore challenge is inoculated directly onto the items. End users should determine the populations of inoculated items. Manufacturers should place indicators within loads at locations believed to be hardest for the agent to reach, on the basis of visual examination.

Process Confirmation/Microbiological Challenge

The core of the validation activity is confirmation of acceptable process parameters and inactivation of the microbial challenge. The end user should expect a linear death curve for the spore challenge and require total death of the challenge. The end user can consider adjustment in chemical sterilant concentration, process time, agitation, and other factors. Proof of cycle efficacy is provided in replicate studies in which the biological indicators are killed, and physical measurements are taken as documentation.

Agent Neutralization/Removal

After exposure, the sterilizing agent must be adequately removed from the items or neutralized before further processing. This segment of the process uses chemical neutralization or physical removal and must be executed in a manner that preserves the sterility of the items. Aseptic processing with appropriate capability demonstration should be provided. Process simulation beginning with the completion of sterilization through placement into a sealed sterile container is expected. See *Sterility Assurance* ⟨1211⟩ for additional information.

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Routine Process Control

Liquid-phase sterilization must be subject to controls that maintain the validated state. The practices outlined in *Sterilization of Compendial Articles* ⟨1229⟩ address the general requirements for all sterilization systems. Sterilization is accomplished by means of a number of related practices that are essential for continued use of the process over an extended period of time, including calibration, chemical and physical measurements, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

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⟨1229.7⟩ GASEOUS STERILIZATION

INTRODUCTION

The use of sterilizing gases for the preparation of materials and equipment is commonly used for items that are susceptible to damage by heat or radiation processes. Many polymeric materials, especially medical devices, are surface sterilized in this manner, as is nonpressure-rated process equipment. The sterilization of dry powders using gases is inappropriate due to the inability of gases to penetrate solid materials. The majority of gas sterilization processes employ ethylene oxide (EO), and procedures for use with other gases generally are patterned after EO practices. Ozone, mixed oxides of nitrogen, and chlorine dioxide are some of the other gaseous sterilants used. [Systems that can exist in liquid and gas phase at the operating temperatures (e.g., hydrogen peroxide, peracetic acid, and paraformaldehyde) are excluded from consideration in this chapter.] EO's ability to penetrate through polymers, cellulosics, and other materials allows it to be used for the terminal sterilization of medical devices in their final packaging. The other sterilizing gases may be suitable for similar applications.

Process control for gas sterilization equipment is accomplished by control of sterilant gas concentration, relative humidity, temperature, and system pressure. Mixing of the gas in the sterilization chamber may be beneficial. EO sterilization may be used for parametric release as described in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩.

Gas sterilization differs markedly from processes during which the agent used can condense during the operation. Vapor sterilization processes will be addressed separately in *Vapor-Phase Sterilization* ⟨1229.11⟩.

As outlined in *Sterilization of Compendial Articles* ⟨1229⟩, analysts must take care in ensuring sterility and demonstrating that the essential quality attributes of the materials are not adversely affected by the process. With respect to gas processes, key considerations include the immediate effects of sterilizing gas on the materials or equipment being sterilized, residual sterilant, sterilant byproducts, and potential chemical reactions. The common gas processes differ slightly with respect to process execution and material concerns and thus are described individually.

ETHYLENE OXIDE

EO is a powerful alkylating agent that destroys microorganisms by chemical reaction, primarily with cell DNA. The destructive mechanism largely follows first-order kinetics and depends on concentration, humidity, and temperature. The use of EO for medical devices in their final packaging has, to a large extent, shaped EO sterilization processes (and, to a lesser extent, all gas sterilization) for other applications (*2,3*). The usual EO process follows a sequence of prehumidification, air removal, rehumidification in the chamber, gas exposure, gas removal from the chamber, and postexposure aeration. The preexposure steps ensure that adequate moisture is present on and within the items being sterilized. The postexposure steps provide time for the diffusion of EO and its byproducts out of the materials and packaging. When EO is used for nonporous equipment the process can be streamlined, which eliminates many of the pre- and postexposure steps because of the need only for surface sterilization. During EO sterilization the gas is introduced at the beginning, and only minimal additions are necessary later to maintain pressure as the gas is absorbed into the material/sterlization load within the vessel. Humidity adjustment during the process also may be required. In some instances, EO reacts with materials in the load to form ethylene chlorohydrin and ethylene glycol. These compounds, including EO, must be reduced to safe levels before the items can be used by patients (*4,5*). EO processing requires strict worker safety and environmental controls because it is associated with carcinogenicity, mutagenicity, and neurotoxicity. In addition, EO is explosive in concentrations of greater than 2.6% by volume in air, therefore, inert gases are often used to minimize flammability. The commonly accepted biological indicator (BI) strain is *Bacillus atrophaeus* (formerly *B. subtilis* var. *niger*).

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OZONE

Ozone is a potent oxidizing agent produced by passing a stream of oxygen or air through a high-voltage electrical field. Ozone is an effective biocidal agent for treatment of water supplies and has demonstrated lethality at concentrations from 2%–10% in air. Optimal microbial destruction is accomplished when the relative humidity is above 80% at room temperature. Ozone degrades to oxygen in the presence of moisture and metals and therefore usually is generated in situ. Ozone does not penetrate porous materials to the same extent as EO does. Process systems that use ozone for gas sterilization have the advantage of simplicity. Its generation and destruction (using a catalytic converter) are accomplished without moving parts or consumables other than the supplied oxygen. The sterilization process uses a sequence of humidification, injection, exposure, and ventilation to remove the ozone from the chamber at the end of the cycle. The common BIs identified for ozone are *Geobacillus stearothermophilus* and *Bacillus atrophaeus*.

CHLORINE DIOXIDE

Chlorine dioxide is an effective sterilizing gas. Pure chlorine dioxide is metastable and therefore is generated as needed. Chlorine dioxide is noncarcinogenic, nonflammable, and effective at ambient temperatures. Its ability to penetrate materials may be less than that of EO.

A typical chlorine dioxide sterilization process uses a sequence of preconditioning, conditioning dwell period, charge, and exposure, followed by aeration.

The BI most commonly used is *Bacillus atrophaeus*.

NITROGEN DIOXIDE

Nitrogen dioxide is a sterilizing gas effective at ambient temperature. Liquid nitrogen dioxide is converted to a gas on introduction to the target chamber. Nitrogen dioxide is nonexplosive and its residues are noncarcinogenic, noncytotoxic, and nonteratogenic. It has a limited ability to penetrate polymeric materials in comparison to EO, which makes postcycle aeration more rapid. It is incompatible with cellulosic materials such as paper and cardboard. The suggested BIs for nitrogen dioxide are *G. stearothermophilus* and *B. atrophaeus*.

VALIDATION OF GAS STERILIZATION

The validation of gaseous sterilization generally begins with the establishing of a "minimum lethal process dwell time" through the use of fractional exposure studies. These fractional studies establish that exposure time, under standard process conditions, where the biological indicator is fully inactivated. This minimum exposure time then becomes the basis for the application of the half-cycle approach for validating the sterilization cycle. The absence of information relating the effect of varying gas concentration, humidity, and temperature on microorganisms resulted in a conservative assumption that the bioburden is equal in antimicrobial resistance and population to that of the biological indicator. The half-cycle method can be defined as follows.

The half-cycle validation method requires the destruction of a high concentration (NLT 10^6 spores) of a resistant microorganism under defined, minimum conditions for complete kill. This establishes the minimum lethal process dwell time. In routine operation, the process dwell period is arbitrarily doubled and supports a theoretical reduction of the biological indicator (and thus the bioburden) to a probability of a nonsterile unit (PNSU) of 10^{-6} (for definitions of terms in this chapter, see *Sterilization of Compendial Articles* ⟨1229⟩).

The half-cycle method used for gas sterilization is shown in *Figure 1*.

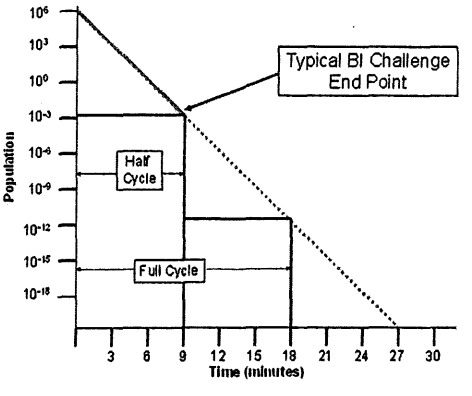

Figure 1. Half-cycle sterilization validation.

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Alternative approaches to cycle validation are available. Gillis and Mosley developed a means for parametric evaluation of EO sterilizing conditions that may result in greater use of other validation methods (*6*). A bracketing approach (see *Figure 2*) that better supports the process operating ranges for the critical parameters relative to the half-cycle method has also been used. In the bracketing method, one evaluates conditions that bracket the defined process condition in order to establish parameters for the minimum and maximum effects on the materials and bioburden. The minimum lethal process dwell time (see half-cycle description above) establishes the worst case for microbial kill. Incremental increases in process dwell time beyond the minimum lethal process dwell time are used to establish the routine and maximum exposure periods, the latter of which imparts the greatest effect on materials. In addition, adjustments to agent concentration and relative humidity are utilized to further enhance the bracketing approach. By this method, the routine process conditions may be established between the minimum and maximum process conditions to assure complete microbial kill while maintaining the integrity of the materials.

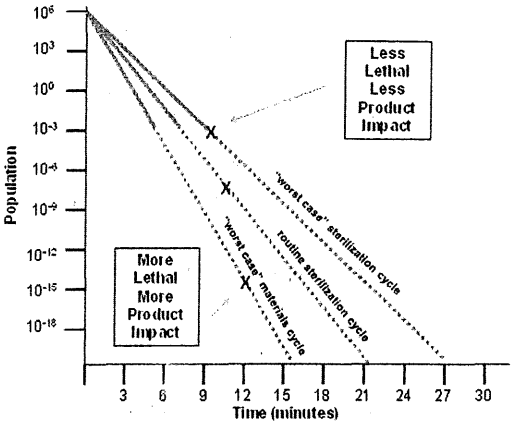

Figure 2. Bracketing method.

Equipment Qualification

The equipment qualification for gas sterilization should include both pre- and postcycle systems to confirm that the equipment has been properly installed and operates as intended.

Empty Chamber Parameter Distribution

Despite the use of true gases, evaluation of parameter uniformity across the chamber is a common activity. This ensures that the gas and humidity introduction methods provide consistency throughout the chamber and can be correlated to the routine monitoring location(s), when present. Biological indicators are not required in the evaluation of the empty chamber uniformity.

Component and Load Mapping

Component and load mapping using invasive sampling are not a part of gas sterilization because sampling systems placed within the load items would alter gas and humidity penetration. Evaluation of lethal conditions with individual items and across loading patterns is best provided by biological indicators or process challenge devices placed within the load items and distributed within the load. Indicators or process control devices are placed within the items and load at locations believed to be hardest for the gas and humidity to penetrate.

Biological Indicators

The biological indicator of choice for gas sterilization varies, as noted above. *B. atrophaeus* (ATCC 9372) is used with EO and chlorine dioxide, and ozone sterilization is monitored with *G. stearothermophilus* (ATCC 12980 or 7953). D-values for the biological indicator can be used to establish exposure periods for the sterilization process to ensure adequate process efficacy. When positioning biological indicators within items it is important to ensure that the placement of the BI does not occlude gas passage or otherwise interfere with the distribution/penetration of the sterilant within the item.

Process Confirmation and Microbiological Challenge

The core of the validation activity is the confirmation of acceptable process parameters with simultaneous physical and chemical measurement and microbial challenge. Sensors are placed in the chamber, or biological indicators are positioned within the load items. Proof of cycle efficacy is provided in replicate studies in which the biological indicators are killed and the physical measurements correspond to the expected values.

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ROUTINE PROCESS CONTROL

Gas sterilization is subject to formal controls that maintain a validated state over time. The practices outlined in ⟨1229⟩ include the general requirements appropriate for all sterilization systems. Sterilization is accomplished by a number of related practices that are essential for continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, ongoing process control, change control, preventive maintenance, periodic reassessment, and training. When parametric release has not been established, biological indicators positioned within the load are used for routine release of each sterilization load, along with a review of documentation from the sterilizer control system.

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⟨1229.8⟩ DRY HEAT STERILIZATION

Dry heat sterilization is a process utilized for heat-stable items (glass, stainless steel, nonaqueous liquids, powders, etc.) that are unsuited for steam sterilization because of either an absence of water (nonaqueous liquids and powders) or requirements for absolute dryness following processing (product contact parts for nonaqueous products). Because dry heat relies on air for the transfer of heat to and from the load items, the process takes longer than a steam process for a comparable size item or load. Lengthy heating and cooling periods require that the load items be unaffected by heat over a long period of time and also require the use of the overkill method for cycle development and validation.

Dry heat sterilization is typically performed in the range of 160°–190° where the objective is sterilization rather than depyrogenation. (Depyrogenation will be covered separately in *Dry Heat Depyrogenation* ⟨1228.1⟩). In dry heat sterilization, hot air is in direct contact with the load items (whether wrapped or unwrapped) and transfers some of its thermal energy. Unlike steam sterilization, in dry heat sterilization there is no phase change of the heating medium, and thus heat transfer is less efficient. The items can be stainless steel, glass, ceramic, or other heat-stable materials and may be wrapped or covered with aluminum foil to protect them during pre- and postprocess handling. Dry heat sterilization is commonly used for heat-stable materials (e.g., petrolatum or powders).

The limited heat transfer capacity of air requires that items in the oven be placed in locations that were confirmed to be acceptable during the validation effort. Manufacturers should exercise caution with varying load sizes because in some instances (resulting from system design and control probe positioning) minimum load sizes may present a worst case.

STERILIZATION CYCLE CONTROL

Process equipment for dry heat sterilization is controlled by calibrated temperature sensors. During the exposure portions of the cycle, attainment of a minimum dwell time at a predefined temperature is used to document process lethality. Cycle efficacy for dry heat sterilization customarily is measured using F_H, which typically is defined as the amount of time the load receives the equivalent of exposure at 170°. The F_H approach is used to compare sterilization processes that operate at varying temperature conditions to a single standard. The process lethality at temperatures other than 170° can be calculated to determine lethality equivalent to that provided at 170°. Sterilizer control systems must deliver conditions within a predefined time–temperature or F_H range. Simple mathematics can be used to calculate the total lethality over the course of the process. For the specific reference temperature of 170° and a *z*-value (for definitions see *Steam Sterilization by Direct Contact* ⟨1229.1⟩) of 20°, the F_H calculation can be determined by the following equation:

$$F_H = \int_{t_1}^{t_2} 10^{\left(\frac{T-170}{20}\right)} dt = \sum_{t_1}^{t_2} 10^{\left(\frac{T-170}{20}\right)} \Delta t$$

F_H = accumulated lethality
t_2 = end time
t_1 = start time

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T = temperature

Accumulation of the lethality (F_H) for the sterilization process across the entire cycle (heat-up and cool-down segments included) includes the contribution of those segments and allows the cycle to be defined by a targeted lethality rather than by a time at a defined minimum temperature.

VALIDATION OF DRY HEAT STERILIZATION

Because dry air has limited heat capacity and dry heat conditions are more variable than those encountered with other thermal sterilization methods, analysts routinely validate their dry heat sterilization procedures using the overkill method as defined in *Sterilization of Compendial Articles* ⟨1229⟩.

Overkill sterilization can be defined as a method in which the destruction of a high concentration of a resistant microorganism supports the destruction of reasonably anticipated bioburden that could be present during routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum lethality; a defined set of method conditions; or confirmation of minimum log reduction of a resistant biological indicator.

The validation requirements for the overkill method are less onerous than those of the other sterilization approaches. Because the load items can withstand substantial amounts of heat without adverse consequence, the greater lethality provided by the overkill method clearly is justifiable.

Equipment Qualification

Equipment qualification is a predefined program that focuses on the sterilizing equipment to confirm that it has been properly installed and operates as intended before evaluation of the sterilization process. In some companies, equipment qualification is separated into installation qualification and operational qualification or is lumped together under a joint terminology of installation/operational qualification. The major use of qualification of the sterilizing equipment is to provide a baseline for preventive maintenance and change control, ensuring reproducibility of operation over time and assurance that the sterilization process is constantly and accurately performed.

Empty Chamber Temperature Distribution

The equipment should be evaluated for empty chamber temperature distribution. The oven or tunnel should be evaluated to determine the range of temperatures within the system, and the cycle parameters should be determined to ensure adequate lethality across the expected load. The acceptance criteria for empty chambers can vary with the equipment capabilities and customary use, but it is typically less uniform than observed in steam sterilizers. Biological indicators are not required during the evaluation of empty chamber temperature distribution.

Component Mapping

Load items that are complex and feature enclosed volumes and product contact surfaces should be subjected to component mapping to determine internal cold spots. This is particularly important in powder sterilization. For each load item, manufacturers should establish the ability of heat to penetrate the items or containers and to bring them to the required temperature. These studies can be performed in a laboratory setting and need not be repeated when the same item is sterilized in other equipment. Thermocouples should be placed into direct contact with the item(s) being evaluated. During component mapping load items should be prepared and oriented in a manner that is consistent with how they will be processed.

Load Mapping

Fixed loading patterns for dry heat sterilization in batch ovens are preferable because the limited heat capacity of the air allows substantial temperature differences across the load. It may be possible to validate maximum and minimum loads as determined by either the number of items or their mass within the oven. Loading in a continuous tunnel process is typically well defined by the limitations of the conveying system. Load and component mapping ensures that all load items attain the required temperature. Information from the load mapping is used to adjust cycle timing to ensure appropriate lethality. System control must consider the relationship between load position and size relative to temperature control locations.

Biological Indicators

The biological indicator (BI) for dry heat sterilization is *Bacillus atrophaeus* (ATCC 9372), a thermophilic spore-former with high resistance to dry heat. The spore challenge is placed on a substrate positioned within the load or on a load item. If spores are used as intended by the BI manufacturer, the population and resistance information provided by the vendor can be used. End users should determine the population and resistance of their biological indicator used when inoculating their own items.

Heat Penetration and Microbiological Challenge

The core of the validation activity is the confirmation of acceptable heat penetration using temperature measurements and microbial challenges. Thermocouples and BIs are placed within the load items at the locations determined during the component and load mapping to present the worst case. Thermocouples should be placed into direct contact with the item(s) being monitored. Proof of cycle efficacy is provided by replicate studies in which the BIs are killed and the physical measurements

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correspond to the expected values of time–temperature or F_H. If the microbial and physical measurements do not correlate, manufacturers should conduct an investigation and should take corrective action to rectify the discrepancy. This study customarily is performed slightly subminimal to the lower specification limits for time, temperature, and/or cumulative lethality.

ROUTINE PROCESS CONTROL

As with all sterilization processes, after the dry heat sterilization process has been validated, it must be subject to formalized controls that keep it in a validated state over time. General chapter *Sterilization of Compendial Articles* ⟨1229⟩ details the general practices that are appropriate for all sterilization systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, physical integrators and indicators, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

REFERENCE

1. USP General Chapters—Microbiology Expert Committee. An outline of planned changes to *USP Sterility Assurance* ⟨1211⟩. *Pharm Forum.* 2012; 38(2).

⟨1229.9⟩ PHYSICOCHEMICAL INTEGRATORS AND INDICATORS FOR STERILIZATION

INTRODUCTION

Physicochemical integrators provide some assessment of sterilization process efficacy and may be used in cases where validation of a sterilization process is not required—an exception is the validation and monitoring of radiation sterilization with dosimetry. The physicochemical indicator provides an immediate visual confirmation that an item has been exposed to a sterilization process. Performance standards both within and between lots of physicochemical integrators or indicators from a given manufacturer should be consistent. Integrators or indicators should not interact physically or chemically with any container or product when placed in the sterilizer load, and should not alter the strength, quality, or purity of the sterilized article. The integrator or indicator should be positioned such that it does not alter the effectiveness of the sterilization process. The principal usage of physicochemical integrators and indicators is to provide a rapid means of confirmation of sterilization cycle completion. This is especially important with single door sterilization chambers where a potential mix-up of nonsterilized and sterilized items is more likely. Aside from radiation sterilization where dosimetric data is accepted as definitive they should not be used as the sole proof of cycle efficacy (see *Radiation Sterilization* ⟨1229.10⟩).

PHYSICOCHEMICAL INTEGRATORS

A physicochemical integrator is defined as a device that responds to one or more sterilization process critical parameters, which results in a measurable value that can be correlated to microbial lethality. The manufacturers of physicochemical integrators should provide data to demonstrate that the labeled performance characteristics tests of the integrators are met.

Physicochemical integrators require precautions for use and the appropriate interpretive criteria to define their performance characteristics. Performance of the sterilization apparatus must be ascertained from records generated by calibrated instruments (temperature, pressure, exposure time, gas concentration, and others, as applicable). The integrator can demonstrate only inadequate or adequate exposure to a combination of sterilization parameters.

Physicochemical integrators for radiation sterilization are designed to react predictably to the delivered radiation dose and can provide primary evidence of sterilization process effectiveness. The use of dosimeters in radiation sterilization cycle development and routine process control is addressed in ANSI/AAMI/ISO 11137-3, Sterilization of health care products—Radiation—Part 3: Guidance on dosimetric aspects (*1*).

PHYSICOCHEMICAL INDICATORS

A physicochemical indicator is defined as a device that provides visual evidence of exposure to one or more critical sterilization parameters. Physicochemical indicators cannot provide primary evidence of sterilization efficacy.

REFERENCE

1. ANSI/AAMI/ISO 11137-3:2006/(R)2010, Sterilization of health care products—Radiation—Part 3: guidance on dosimetric aspects. New York: American National Standards Institute; 2010.

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⟨1229.10⟩ RADIATION STERILIZATION

INTRODUCTION

Radiation sterilization utilizes the lethal effect of various forms of radiation as a means of microbial destruction. Ionizing radiation (gamma, x-ray, or beam) sterilization is used extensively for the sterilization of medical devices and for a variety of other materials and products. Nonionizing sterilization methods such as microwave, infrared, x-ray, and ultraviolet light may be useful but have more restricted application, and are outside the scope of this chapter. This chapter provides an overview of sterilization using ionizing radiation and its validation, including dose setting, material compatibility, and dose verification.

The effects of radiation on materials can be substantial and are a major consideration when manufacturers select radiation as a processing method. The advantages of sterilization by irradiation include simplicity, absence of mechanical complexity, reproducibility, and overall efficiency. In fact, radiation sterilization is unique because the basis of control essentially is the absorbed radiation dose, which can be precisely measured. Methods used to establish appropriate radiation doses to achieve the desired sterility assurance level are defined in ISO 11137-1 Sterilization of Health Care Products; ISO 11137-2 Sterilization of Health Care Products—Radiation—Part 2: Establishing the Sterilization Dose; and ISO TS 13004: 2013 Sterilization of Health Care Products—Radiation—Substantiation of Selected Sterilization Dose: Method VDmaxSD. These methods include Method 1, Method 2A, Method 2B, and Method VD_{max}, which differ in the specific testing scheme and the number of articles that are needed for testing and are based on certain assumptions about bioburden. The use of a biological indicator is inappropriate during radiation sterilization validation because (a) there are accurate correlations between dose measurement and microbial destruction for a wide range of microorganisms and (b) the established dose setting methods are based on the material's bioburden in its natural state. These correlations have been developed by the medical device industry and provide a direct methodology for process control. Dosimetry plays a central role in radiation sterilization and serves as a direct means for affirming process lethality. The radiation dose measured in kGy (formerly MRads) is directly related to the lethal effects of the radiation on microorganisms. The measured dose has the same utility as F_0 in steam sterilization. Routine process control for radiation sterilization is provided by one or more reference dosimeters on the exterior of the packages (after dosimeters have been correlated during validation with dose measurement inside the package). The robustness and reliability of the absorbed dose of the article to be sterilized can support parametric release, as described in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩, for many items.

GAMMA STERILIZATION

Gamma sterilization entails the use of a specifically designed facility where items to be sterilized are exposed to a Co^{60} radiation source in a manner that ensures uniform dosing. Highly penetrating photons (gamma rays) are emitted from Co^{60} as it decays to Ni^{60}. The half-life for this isotope is 5.27 years, which means that over the course of each year the source loses about 12% of its radioactivity. This steady reduction in radioactivity requires that radiation process operators adjust their process controls (typically exposure time) to maintain the established dose required. Periodically, additional Co^{60} is required to maintain practical throughput.

X-RAY STERILIZATION

X-ray sterilizers generate highly penetrative photons similar to the gamma photons from Co^{60} irradiators. X-ray photons are generated when accelerated electrons impact a target such as tantalum. These systems rely on scanning of materials with x-ray photons in order to sterilize them. Properly maintained, these systems are able to deliver a constant dose over time. No local radioactive source is required for x-ray sterilization systems.

E-BEAM STERILIZATION

Electron beam systems rely on scanning of objects with focused electrons to sterilize the items within a defined radiation field. Properly maintained and controlled, these systems deliver a constant dose, so there is no change in dose with respect to time. The principal advantages of electron beam sterilization are a much higher dose rate and the absence of a localized radioactive source. These systems can be installed and operated by the end user. Electron beam penetration is substantially less than that obtained with photons, and therefore dose mapping is critical to ensure that items of varying density and complexity are properly sterilized. Because of the high dose rates used with electron beam sterilization, some materials can experience significantly higher temperatures than the same materials would experience in Co^{60} irradiation.

VALIDATION OF RADIATION STERILIZATION

Cycle development for radiation requires the identification of an appropriate radiation dose for the objects and confirmation that the dose does not adversely affect the material's essential quality attributes. In other words, analysts should identify the minimum sterilization dose as well as the maximum dose the material can withstand without negative effects. With this information analysts can set the dose for a specific radiation sterilization application.

Dose setting or dose establishment typically is achieved by following one of the ISO methods. These are Method 1, Method 2A, Method 2B, and Method VD_{max}. The choice of the most appropriate method depends chiefly on production batch size,

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knowledge of the normal bioburden, and the material's sensitivity to radiation. Dose mapping plays an important role through the cycle development and dose-setting exercise.

Dose Setting

Method 1 is based upon the assignment and verification of a sterilization dose based on a microbial population. The resistance of the microbial population is not determined, and dose setting is based on a standard radiation resistance assigned to the microbial population, derived from data obtained from medical device manufacturers and from the literature. This analysis assumes that the distribution of standard resistance represents a more severe challenge than the natural microbial population on the material to be sterilized. A verification dose study should confirm the relative resistance assumption. The VD_{max} method is similar to Method 1 (it requires both bioburden and dose verification testing) but relies on bioburden ranges (e.g., <1000 CFUs per item for a 25-kGy sterilization dose and, for example, 0.1–1.5 CFUs per item for a 15-kGy sterilization dose).

The more complex Method 2 does not require the enumeration of the microbial population for the purpose of setting the sterilization dose (although it is required for routine monitoring and control) but uses a series of incremental dose exposures to establish a dose at which approximately 1 out of 100 samples irradiated at that dose will be nonsterile. This is not the sterilization dose, but it provides the basis to determine the sterilization dose by extrapolation from this information.

Material Compatibility

Once the required dose level has been established, the maximum dose should be established. Analysts typically establish the maximum dose by evaluating the highest likely dose that might be seen during the sterilization process, adding a safety factor, and evaluating the item for immediate and long-term effects of the radiation exposure. Some materials may appear unchanged initially, and the effects may become evident only over time. The evaluation should consider all of the materials exposed to the radiation processing, especially the drug product and its primary container. Product stability, safety, and functionality should be confirmed over the product's intended use period.

Dose Verification

The methods for cycle development and dose setting rely on the bioburden approach. Analysts use defined presterilization bioburden controls and periodic evaluation of the process effects on the bioburden to maintain cycle efficacy. Establishing the required dose for microbial destruction during cycle development uses the bioburden's natural resistance; analysts then extrapolate the dose-setting algorithms to establish a dose that is capable of delivering a probability of a nonsterile unit (PNSU, a standard measurement) of 1×10^{-6}. The results of the dose-setting approaches for initial bioburden with different populations and resistance to radiation sterilization are depicted in *Figure 1*.

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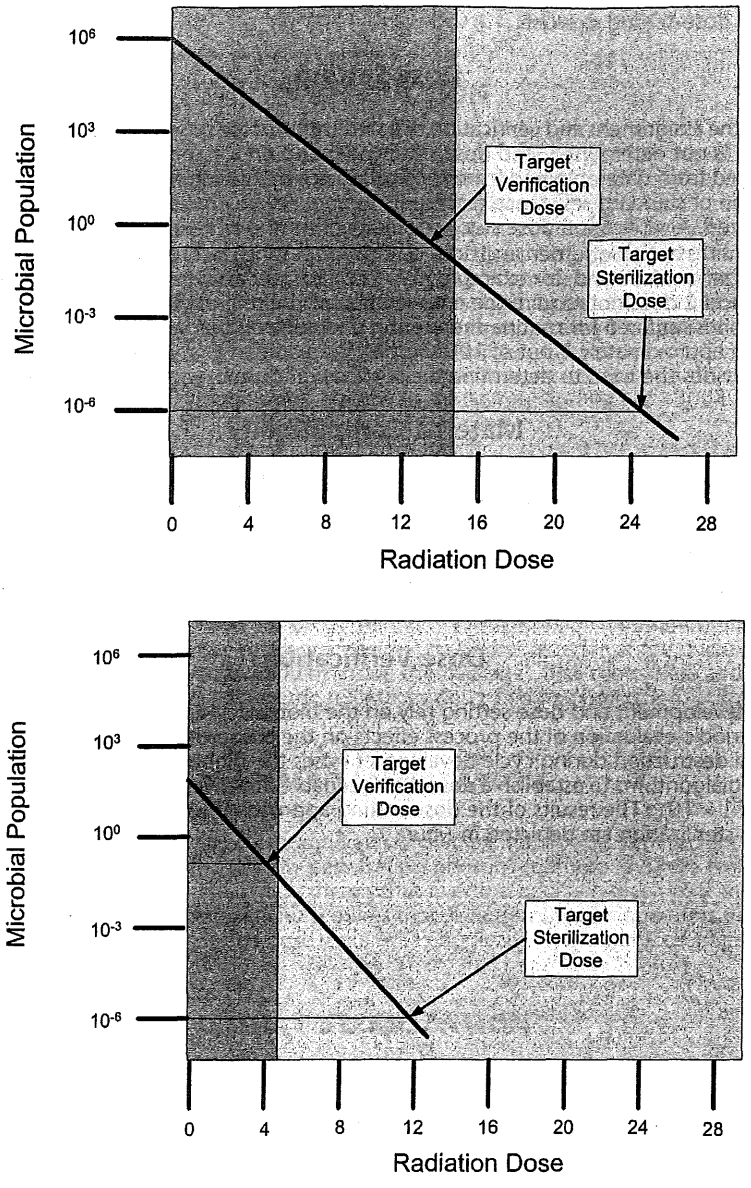

Figure 1. Results of radiation dose setting using VD_{max}. (top) Higher bioburden population, higher resistance to radiation sterilization. (bottom) Lower bioburden population, lower resistance to radiation sterilization.

Validation Activities

Confirmation of appropriate dose delivery when using the sterilization dose requires a number of supportive activities.

EQUIPMENT QUALIFICATION

The use of gamma sterilization requires initial and periodic assessment of equipment controls and parameters necessary to establish the system's capability. Sterilization systems that deliver directed beams or rays have controls for scan speed, source intensity, and system timers. The other elements of radiation sterilization equipment largely are related to material transport and are easily qualified. Qualification of safety controls, devices, and software is required.

EMPTY CHAMBER DOSE MAPPING

This optional exercise entails mapping the target area for radiation dose in the absence of a load and is a possible means to evaluate a focused beam or ray system. It provides a baseline of performance that may be useful over time.

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LOAD DOSE MAPPING

The arrangement of items in irradiation containers, carriers, or pallets is an essential part of the initial validation exercise. The goal of the mapping is to define the distribution of a dose throughout the load items and establish a configuration that minimizes dose variation across the materials. The items are mapped using multiple dosimeters positioned internally and externally. Identification of maximum dose location is important in evaluating the effects of the radiation on the load items. The location of minimum and maximum dose can be identified from the dose mapping data for monitoring in routine sterilization of materials.

BIOLOGICAL INDICATORS

The use of biological indicators for radiation sterilization is not indicated because the physical and dosimetric measurements employed are more reliable, reproducible, and robust than biological systems.

DOSIMETRY

Process control for radiation sterilization relies heavily on dosimetry for both initial development and ongoing verification. For guidance on the selection and use of a dosimetry system for use in radiation sterilization refer to ASTM E2628 Practice for Dosimetry in Radiation Processing. The dosimeters and the instruments used with them should be calibrated according to ISO/ASTM 51261 Practice for Calibration of Routine Dosimetry Systems for Radiation Processing.

PROCESS CONFIRMATION

The core of the validation activity is the confirmation of acceptable lethality using dosimeters that are positioned across the material as it is processed through the radiation-sterilizing equipment. Proof of sterilization cycle efficacy is provided in replicate studies in which the dosimetry results correspond to the required minimum value for sterility assurance and demonstrate that the maximum value has not been exceeded.

Routine Process Control

Radiation sterilization should be subject to formal controls that maintain the validated status. The practices outlined in *General Principles of Sterilization of Compendial Articles* ⟨1229⟩ provide the general requirements appropriate for all sterilization systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The practices that are essential to maintain validated status for radiation include training, calibration, physical measurements, bioburden monitoring, change control, preventive maintenance, and periodic dose audits.

REFERENCES

1. Jacobs, G., Validation of the Radiation Sterilization of Pharmaceuticals, chapter in Agalloco, J., & Carleton, F. J. (eds.), *Validation of Pharmaceutical Processes*: 3rd Edition, InformaUSA, New York, 2007.
2. Herring, C., & Saylor, M., Sterilization with Radioisotopes, chapter in Morrissey, R., & Phillips, G. B. (eds.), *Sterilization Technology—A Practical Guide for Manufacturers and Users of Health Care Products*, Van Nostrand Reinhold, New York, 1993.
3. Cleland, M., O'Neill, T., & Thompson, C., Sterilization with Accelerated Electrons, chapter in Morrissey, R., & Phillips, G. B., *Sterilization Technology—A Practical Guide for Manufacturers and Users of Health Care Products*, Van Nostrand Reinhold, New York, 1993.

⟨1229.11⟩ VAPOR PHASE STERILIZATION

INTRODUCTION

Sterilization can be accomplished using sporicidal agents suspended in air (i.e., vapor). Sterilizing agents that operate in this fashion include hydrogen peroxide (H_2O_2), peracetic acid (CH_3CO_3CH), formaldehyde (CH_2O), and glutaraldehyde [$CH_2(CH_2CHO)_2$] in aqueous solution. At room temperature these are liquids or solids that can be vaporized for introduction into a vessel or chamber. They differ from sterilizing gases and liquids in that there are multiple phases within the vessel during sterilization. Vapor sterilization systems are well suited for heat-sensitive materials and surface sterilization. Items exposed to the process should have their surfaces exposed to the greatest extent possible. Vapor sterilization processes require appropriate sterilant concentration, temperature, and relative humidity, all of which may be variable during the exposure period. Because the agent is ordinarily supplied as an aqueous solution, moisture is introduced with the agent. The consequences of variation in these parameters may be localized differences in relative humidity, agent concentration, and condensation rates on the surfaces to be treated, resulting in variations in process lethality. The parameters to be established include sterilant amount (usually derived from injection quantities), relative humidity, and temperature. There is no demonstrated correlation between gas phase conditions, surface conditions, and microbial kill. For this reason, online monitoring of vapor phase concentration is not widely utilized as a control parameter. Efforts to develop a standardized biological indicator for vapor systems have been hampered by the multiphasic nature of these sterilants. Selection of the appropriate biological indicator (BI) and resistance

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should be based on experimentation within the user's system. Only under well-defined, specific conditions (e.g., agent concentration, humidity level, temperature, substrate, and phase) can a reliable D-value be established (for a definition of D-value, see *Sterilization of Compendial Articles* ⟨1229⟩).

This chapter will briefly review hydrogen peroxide and peracetic acid sterilization systems, because they are more widely used than other vapor phase sterilizing agents in the pharmaceutical and medical device industries.

HYDROGEN PEROXIDE

The efficacy of hydrogen peroxide as a liquid sterilant has been long established.[1] There are several effective approaches to hydrogen peroxide (H_2O_2) injection, including continuous, intermittent, or all at once. Some of the systems utilize an evacuation or drying step prior to introduction of the hydrogen peroxide (H_2O_2) to allow for increased concentration without excess condensation. Alternatively, hydrogen peroxide (H_2O_2) can be introduced as a liquid, followed by target heating. Following the exposure period, the chamber or target is aerated to an acceptable level for further processing of materials and/or personnel exposure (whichever is lowest) prior to opening and removing the sterilized article.

PERACETIC ACID

Peracetic acid (CH_3CO_3CH), alone or mixed with hydrogen peroxide, is a sporicidal agent that has been proven effective.[1] Peracetic acid is introduced as a liquid through an atomizer, resulting in the presence of liquid and vapor phases within the chamber. After the process dwell period, it is removed by evaporation.

VALIDATION OF VAPOR STERILIZATION

Standard sterilizing conditions have not been defined due to the varying phase and multiphase nature of the sterilant during sterilization processes. Therefore, no standardized BIs with D-values that may be used for conventional predictive analysis of kill rates exist. In the absence of BI D-values, and due to the variation in vapor sterilization cycle parameters, an empirical approach must be used. The kill rates in the gas and liquid phases that constitute the vapor may be substantially different (liquid kill rates are considered greater than gaseous kill rates). Destruction of BIs distributed across the system or load demonstrates lethality regardless of which phase effects the kill. Sterilization process parameters (usually time) that do not kill the BIs may be adjusted until a complete kill is achieved. This establishes the minimum conditions necessary for a complete kill. Vapor sterilization may be validated using a half-cycle, bracketing, or other suitable approach as defined in ⟨1229⟩ and *Gaseous Sterilization* ⟨1229.7⟩.

The half-cycle validation method requires the destruction of a suitable concentration of a resistant microorganism under defined, minimum conditions for a complete kill. Then, in routine operation, the minimum lethal time period is arbitrarily doubled, which supports a doubling of the spore log reduction of the BI, and is more than sufficient to inactivate the bioburden.

A bracketing approach, which better supports process operating ranges for the critical parameters than does the half-cycle method, also can be employed. In the bracketing method, one evaluates conditions that bracket the defined process condition in order to establish parameters for the minimum and maximum effects on the materials and bioburden. The minimum lethal process dwell time establishes the worst case for microbial kill. Incremental increases in process dwell time beyond the minimum lethal time period are used to establish the maximum exposure periods, which impart the greatest effect on materials. In addition, adjustments to the quantity of agent introduced, operating temperature, and relative humidity are utilized to further enhance the bracketing approach. By this method, the routine process conditions may be established between the minimum and maximum process conditions to ensure complete microbial kill while maintaining the integrity of the materials.

The following activities are defined for a batch process, and therefore appropriate adaptation is necessary when they are applied to intermittent or continuous sterilization processes.

- *Equipment qualification*—The equipment qualification for vapor sterilization mimics that of other sterilization processes in order to confirm that the equipment has been properly installed and operates as intended.
- *Empty chamber parameter distribution*—Although multipoint measurement is possible, it lacks correlation to surface microbial kill. Humidity and temperature measurements, along with chemical indicators, can provide a limited indication of sterilant distribution. BIs are not required in the evaluation of the empty chamber.
- *Component mapping*—For surface sterilization, internal mapping of load items is not required. Although vapors are primarily used as surface sterilants, when they are used for packaged articles with internal surfaces and volumes that need to be sterilized, internal mapping should be performed, with BIs placed in difficult-to-penetrate locations to confirm process lethality.
- *Load mapping*—Humidity and temperature measurements, along with chemical indicators, can provide a limited indication of sterilant distribution on component surfaces. BIs are not required. Effects of load size and patterns should be assessed.
- *Biological indicators*—The use of multiple BIs at each test location is recommended to more adequately support the process lethality.
- *Process confirmation and microbiological challenge*—The core of the validation activity is the confirmation of acceptable process parameters and inactivation of the microbial challenge. Proof of cycle efficacy is provided in replicate studies in which the BIs are killed and chemical or physical measurements are utilized.

[1] Block SS, editor. *Disinfection, Sterilization, and Preservation.* 5th ed. Philadelphia: Lippincott Williams & Wilkins; c2001. Chapter 9, Peroxygen compounds; pp. 185–204.

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ROUTINE PROCESS CONTROL

Vapor sterilization is subject to controls that maintain a validated state over time. The practices outlined in ⟨1229⟩ describe the general requirements appropriate for all sterilization systems. The essential practices required to maintain validated status include calibration, physical measurements, use of BIs, physical or chemical integrators and indicators, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

⟨1229.12⟩ NEW STERILIZATION METHODS

INTRODUCTION

Sterilization processes are developed for the elimination of viable microorganisms while preserving the essential physical, chemical, and biological properties of the materials subjected to them. Where this cannot be accomplished by the sterilization methods described in *Sterilization of Compendial Articles* ⟨1229⟩, it may be possible to sterilize by using a proposed method not commonly used. When doing so, it is the end user's responsibility to demonstrate that the proposed new method can be used safely and effectively.

POINTS TO CONSIDER FOR A NEW STERILIZATION METHOD

The major steps in the implementation of a new sterilization method include the following:

- Elimination of any established method through experimental evidence and/or comprehensive literature review of any materials used
- A literature review to identify supportive information on the proposed method
- Identification and confirmation of reproducible lethality against a broad range of microorganisms, including bacterial spore-formers
- Identification and definition of critical process parameters necessary to ensure sufficient lethality. The effective range of these parameters should be explored to identify necessary conditions for the proposed sterilization process. Among the parameters to be considered, depending upon the nature of the process under consideration, are process dwell time, temperature, concentration, energy or power, and relative humidity. This evaluation should include positive and negative controls to ensure that the proposed method is in fact responsible for microbial destruction.
- The selection of a biological indicator (usually a spore-forming microorganism) with increased resistance to the sterilization method
- Evaluation of the proposed method against anticipated bioburden microorganisms and comparison of relative resistance of the bioburden microorganism to that of the chosen biological indicator
- The identification of in-process and/or post-process measurements and/or analysis that can reliably confirm the effectiveness of the proposed sterilization process.

Where the proposed new method is used for materials intended for human and/or veterinary use, the relevant regulatory authorities should be contacted to secure their acceptance before either investigational or clinical usage. Validation of the proposed new method should be completed before use on a commercial basis.

⟨1229.13⟩ STERILIZATION-IN-PLACE

INTRODUCTION

Sterilization-in-place (SIP) can be defined as the sterilization of a system or piece of process equipment in situ. The purpose of SIP[1] is to eliminate, or greatly reduce, the need for post-sterilization handling, including that necessary to make aseptic connections. Mobile process equipment (e.g., portable tanks, storage vessels, and other equipment), once sterilized in this manner, may be relocated. The SIP process can be carried out by using any of the following physical methods: moist heat, dry heat, gas, liquid, or vapor (described below) according to the approaches described in *Sterilization of Compendial Articles* ⟨1229⟩, as adapted for use with the specific equipment or system.

COMMON ELEMENTS OF SIP PROCESSES

There are a number of considerations appropriate for the design and use of SIP procedures that apply to all of the sterilization methods:

- The use of an SIP method is normally associated with a "closed system." Closed systems are almost always sterilized in situ, and the design elements of the typical closed system are consistent with many SIP process needs.

[1] AAMI/ISO 13408-5. Aseptic processing of health care products—part 5: sterilization in place; 2008.

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- For large systems, it may be necessary to sterilize in portions. The individual sterilization processes should overlap to ensure treatment of all internal surfaces.
- The focus of the SIP procedure is sterilization of the product contact surfaces (the interior of the system). Demonstration of process lethality relies upon physical measurements and biological indicators. This confirmation should extend to the "sterile boundary" of the system, including vessel headspace, connections to other vessels/equipment, and other parts of the system. The interior surfaces of the process equipment, irrespective of their materials of construction, should be exposed to lethal conditions sufficient to sterilize the system and confirmed as lethal with an appropriate biological challenge.
- SIP is accomplished almost exclusively using the overkill approach to sterilization. The components of the physical equipment should be chosen based upon their ability to withstand the sterilizing conditions to be used. Filters in the process system, whether membrane or high-efficiency particulate air (HEPA), are typically susceptible to damage during SIP, and care must be taken to preserve their integrity. Filter manufacturers can provide guidance on acceptable sterilization methods and parameters.
- The absence of specifically designed equipment in which the sterilization process is performed places the bulk of responsibility for design onto the user. SIP systems ordinarily cannot be purchased directly in the way one purchases a steam sterilizer or a dry heat oven. Instead, the system, which was designed for the operating process, may require modification to accommodate the SIP process to be used. The user must assume the role of designer for the process, equipment, and control system.
- The system design and operating procedures must provide for an efficient means of introducing and removing the sterilization agent. Sterilization agent removal must consider the potential effects of residual sterilant on the materials to be processed. Establishment of a reliable process sequence is a critical part of the cycle development exercise. The sterilizing agent is normally introduced through a filter on the system that may also serve as a process, purge gas, or vent filter.
- At the conclusion of the sterilization process sequence and until ready for use, the system should be pressurized with a purge gas (sterile air or nitrogen are the most common) to prevent the introduction of contaminants to the sterilized system.
- The critical process parameters for the SIP process should be recorded as the process is executed. The important parameters may include temperature, pressure, concentration, flow rate, humidity, and time, among others.

CLOSED SYSTEMS

In pharmaceutical manufacturing operations, closed systems are used for various applications including maintenance of large quantities of materials (liquids or powders) in a sterile state; manufacture of biological and synthetic organic active ingredients (especially where microbial absence is essential); and preparation of process equipment for use in sterile drug product manufacturing and filling. The use of closed systems provides superior separation of sterile materials from the surrounding environment. Typically, closed systems are maintained under positive pressure at all times. The characteristics of a closed system that establish its designation as "closed" include the following:[2]

- It maintains integrity during all operating periods and under all conditions.
- It is sterilized-in-place or sterilized while closed before use.
- It can be adapted for materials transfer in and/or out while maintaining its sterile state.
- It can be connected to other closed systems while maintaining the integrity of all systems.
- It is subject to scheduled preventive maintenance.
- It uses sterilizing-grade filters for sterilization of liquid and gas process streams.

SIP METHODS

Moist Heat

The use of saturated steam is the most prevalent method for SIP of large systems. The majority of installations use gravity displacement cycles adapted from those originally used in steam sterilizers (the size and complexity of many systems preclude the use of prevacuum cycles). Important considerations include the provision for air removal, condensate discharge, and steam removal post-dwell.[3,4] This method is commonly used for bioreactors, sterile bulk production, holding tank and delivery lines, and other large systems.

Superheated Water

Systems used for *Water for Injection* and *Purified Water* can be sterilized by using superheated water (water that is heated above its boiling point and pressurized to maintain it as a liquid) circulating through the system. This method has the ability to

[2] Parenteral Drug Association, Technical Report No. 28, Revised. Supplement Volume 60, No. S-2, Process simulation testing for sterile bulk pharmaceutical chemicals. Bethesda, MD: PDA; 2005.
[3] Agalloco J. Steam sterilization-in-place technology and validation. In: Agalloco J, Carleton FJ, editors. Validation of pharmaceutical processes. 3rd ed. New York: Informa USA; 2007.
[4] Parenteral Drug Association, Technical Report No. 61, Steam in place. Bethesda, MD: PDA; 2013.

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sterilize vessels, filters, and other wetted components at the same time.[5] Removal of residual water subsequent to the sterilization phase is recommended.

Dry Heat

Dry heat has been used for SIP of spray dryers and their associated material collection systems. The air supply for sterilization in these systems is provided through HEPA filters.

Gas

Gas-phase SIP has been used for non- and low-pressure-rated process equipment, such as freeze dryers, prefreezers, process vessels, and other equipment.

Liquid

Liquid chemical sterilization is best suited for liquid-handling systems and can be used only for fully wetted surfaces. This process is similar to those using superheated water except the lethal modality is chemical rather than thermal.

Vapor

Sterilizing vapors have been used for the in situ sterilization of the same types of process equipment as those treated with sterilizing gases. The precautions associated with vapor sterilization described in *Vapor Phase Sterilization* ⟨1229.11⟩ are required.

ROUTINE PROCESS CONTROL

SIP processes are subject to formal controls that maintain a validated state over time. The practices outlined in ⟨1229⟩ include the general requirements appropriate for all sterilization systems as well as those specific to an individual sterilization method. Sterilization is accomplished by a number of related practices that are essential for continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, physical integrators or indicators, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

⟨1229.14⟩ STERILIZATION CYCLE DEVELOPMENT

INTRODUCTION

The proper development and implementation of a sterilization process requires a number of important sequential steps to ensure that an appropriate sterilization process results in materials and products that are both microbiologically safe and suitable for their intended use.[1] Sterilization technologies that rely on heat, ionizing radiation, and chemicals often have the potential to alter the physicochemical properties of materials to which they are applied. In some cases, sterilization could either leave toxic residues on materials that were sterilized or result in impurities (i.e., leachables) being more readily released into products that contact them. Thus, it is necessary to consider sterilization not only as a means of eliminating microorganisms but also as a process that has the potential to change materials in a manner that impacts usefulness, safety, or both. A balance between the microbial lethality of a sterilization process and other aspects of patient safety must be established. A robust sterilization process that satisfies both is the desired objective of the cycle development activities.

PROCESS SCREENING

Radiation

The methods for establishment of radiation sterilization are described in detail in ISO 11137, and adherence to the most recent version of that standard is recommended.

Sterilizing Filtration

Filtration sterilization processes rely on exclusion of microorganisms and differ substantially from those that rely on lethality (see *Sterilizing Filtration of Liquids* ⟨1229.4⟩). Process development for filtration must consider many factors, such as adverse effects of the fluid on the filter (e.g., chemical compatibility) and of the filter on the fluid (e.g., extractables and leachables), as

[5] Haggstrom M. Sterilization-in-place using steam or superheated water. In: Proceedings of the PDA Basel conference. Bethesda, MD: PDA; 2002.

[1] The term "materials", as used throughout this chapter, includes drug substances, drug products, in-process materials, components, containers, closures, laboratory media, utensils, product contact materials, and other items subject to sterilization processes.

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well as adsorption of fluid components. Physical interactions such as pressure differential, hydraulic shock, flow rate, temperature, and filtration capacity must also be considered. See ⟨1229.4⟩ for additional information.

Other Sterilization Methods

The first step in the development of a sterilization cycle is exposing the materials to the sterilization process and evaluating the impact that the process has on them. Initially, this determination can be a general screening process in which gross changes in appearance may be sufficient to confirm incompatibility. Where appearance is unchanged, the material should be evaluated for changes to its physical and chemical attributes. If the results are marginal, reductions in those parameters that most impact lethality and/or material effects may be beneficial. Tests conducted over a range of process lethality that would result in microbiological destruction should be performed. At this stage of development, the final process conditions required are not yet established, so flexibility in the preliminary evaluation is necessary. Common sense should be applied when considering sterilization process alternatives. For example, thermal treatments are inappropriate for refrigerated materials, items cannot be heated close to their melting point, sterilizing gases and liquids cannot penetrate sealed containers or nonporous materials, and other obvious restrictions may also be present. In the evaluation of items made of many materials (i.e., sealed vials, permeable pouches, etc.), consider the effect on all material components of the item being sterilized. Altering one or more components of the item may result in a favorable outcome. In many projects involving the sterilization of a custom-designed component, the sterilization process evaluation is an essential element of proof-of-principle studies. No component selection and manufacturing process design for a sterile material can be considered complete until a suitable sterilization process has been identified and fully evaluated, and renders the material sterile while having minimal effects on its physical and chemical attributes.

The following conditions are merely examples for consideration when evaluating materials for compatibility with the sterilization processes indicated:

1. Moist heat—at conditions sufficient to reliably kill the expected bioburden (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩ and *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩)
2. Dry heat—check for the lowest melting point of materials and process at 25° below the lowest melting temperature (see *Dry Heat Sterilization* ⟨1229.8⟩)
3. Liquids, gases, and vapors—at conditions sufficient to reliably kill the expected bioburden (see *Liquid-Phase Sterilization* ⟨1229.6⟩, *Gaseous Sterilization* ⟨1229.7⟩, and *Vapor Phase Sterilization* ⟨1229.11⟩)

Other conditions can be utilized in this preliminary evaluation, and multiple methods may need to be explored. Use information in the literature or provided by the suppliers to facilitate this effort. Materials should be changed if the initial materials are suspected of being incompatible with an otherwise acceptable method. The selected sterilization method should be the process that has the least impact on the essential quality attributes of the material and delivers lethality sufficient to exceed the desired minimum probability of a nonsterile unit (PNSU).

BIOBURDEN EVALUATION

In parallel with the process described above, an assessment of the bioburden present in or on the items to be sterilized should be conducted (see *Monitoring of Bioburden* ⟨1229.3⟩). The initial evaluation should include an estimation of bioburden population and resistance. A control strategy for periodic bioburden monitoring for resistance and population should be instituted regardless of the results of the initial study (see ⟨1229.3⟩).[2] For radiation sterilization processes, the expectations for bioburden evaluation are established in ISO 11137.

Bioburden Resistance

For the remaining sterilization processes, the execution of a boil test (100° for 10 min) can serve as a basic screen for spore-forming microorganisms (see ⟨1229.2⟩). The absence of survivors in a boil test greatly simplifies the sterilization cycle development as the sterilizing conditions for non–spore-formers are less stressful on the production materials. If microorganisms are determined to survive the boil test, their resistance to the chosen sterilization process should be established. The boil test allows for estimation of a D value for a moist heat process. For other processes, a literature reference or laboratory study is required (see ISO 18472 *Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment*).

Bioburden Population

If no microorganisms are recovered or only non–spore-formers are recovered, an estimation of bioburden population can be utilized to expedite selection of the appropriate process duration. As spore-forming bacteria are ubiquitous, the potential presence of spores in the bioburden should be given consideration.

[2] Native bioburden must be routinely tested and trended with appropriate screening in place for organisms that may exceed the worst case in terms of population and/or survivability. If a more resistant organism and/or abnormally high population has been isolated through a screening procedure, the impact on the sterilization process to achieve the required sterility assurance level must be determined.

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ESTIMATION OF PROCESS DURATION

The minimum duration of the sterilization dwell period can be determined once the sterilizing method has been selected and the bioburden (as determined or assumed) information has been obtained. This is accomplished by rearranging *Equation 1*, used to estimate the PNSU (see *Sterilization of Compendial Articles* ⟨1229⟩).

$$\log N_U = \frac{-F}{D} + \log N_0 \qquad (1)$$

Rearrangement of the equation and solving for F results in *Equation 2*.

$$F = D\,(\log N_0 - \log N_U) \qquad (2)$$

N_U = assumed PNSU (NMT 1 positive unit in 10^6 units)
F = dwell time of the sterilization process at a defined condition expressed in minutes[3]
D = estimated D value of the bioburden in minutes
N_0 = estimated maximum population of the bioburden

The calculated F value represents the minimum duration of the process dwell period to reduce the assumed initial microbial population to less than the desired PNSU. Safety factors can be added to the assumed D value of the bioburden, the minimum desired PNSU, and the bioburden population estimate. The use of a safety factor reduces contamination risk as it increases the process duration, but it may adversely affect the materials being sterilized. With or without safety factors, determination of the estimated process duration under sterilizing conditions is a prerequisite to the subsequent activities.

Depending on the F value (or process dwell time) selected, the validation approach for sterilization can be determined. The preferred, but not required, method is the use of an overkill approach (see ⟨1229⟩) where the F value allows for complete inactivation of a resistant biological indicator. Where material limitations restrict the use of lengthy sterilizing conditions, the bioburden/biological indicator or bioburden methods may be more appropriate (see ⟨1229⟩).

FORMAL MATERIAL EVALUATION

Once the appropriate sterilization process has been chosen, the effects of that process on the materials should be reconfirmed (*1*). The requirements for this can vary from simple physical examinations for materials expected to be largely unaffected by the sterilization process, such as stainless steel or glass with steam, to comprehensive stability examinations for formulated products in their final container-closure configuration. The criteria for these studies should be objective wherever possible. It is useful to do a literature search for materials about which there is minimal experience, and to search and discuss potential material effects with suppliers. The preparation and sterilization of the test units for these formal studies can be conducted in a laboratory setting and must be fully documented.

When sterilizing using chemical agents, the sterilized materials should be tested for the presence of residual sterilant and its known degradation by-products. Post–sterilization cycle aeration or other treatments may be necessary to reduce these to acceptable levels.

BIOLOGICAL INDICATOR SELECTION

A biological indicator should be selected that is appropriate for the chosen sterilization process and its duration, as well as the intended validation approach (see *Biological Indicators for Sterilization* ⟨1229.5⟩). The chosen biological indicator provides a means to measure the lethality of the process through the selection of biological indicator population and resistance. The sterilization process duration should not be adapted to accommodate the specifics of any biological indicator.

CONTAINER AND ITEM MAPPING

Studies should be performed to determine the location within the materials to be sterilized that is least likely to achieve sterilizing conditions. The mapping can be conducted using thermocouples, dosimeters, biological indicators, and/or chemical indicators placed within the materials. The materials to be sterilized should be wrapped or packaged and oriented as intended for routine use of the sterilization process. The wrapping and packaging materials and methods should be controlled to ensure reproducibility. It is essential when conducting these studies that the introduction of the measuring devices be accomplished in a manner that does not alter (positively or negatively) the penetration of the sterilant through any wrapping and packaging or through the material itself. If the materials to be sterilized have simple geometry, with minimal internal volume, and are sterilized with other more complex items, mapping of the simpler materials can be omitted. Interpolation of volume, density, dimension, and mass may be possible to reduce the extent of studies required. Mapping studies should be documented for future reference.

[3] The F value is equivalent to F_0 for moist heat, F_H for dry heat, or process dwell time L for other sterilization processes and is typically expressed in minutes.

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LOAD MAPPING

The initial use of a sterilization process should include confirmation that the sterilization conditions can be delivered throughout the entire load. Additional guidance on this, and the subsequent aspect of sterilization validation and process control, can be found in other chapters within the ⟨1229⟩ family of chapters.

REFERENCES

1. Association for the Advancement of Medical Instrumentation (AAMI). Compatibility of materials subject to sterilization. Arlington, VA: AAMI Technical Report No. 17; 2008.

⟨1229.15⟩ STERILIZING FILTRATION OF GASES

INTRODUCTION

Gases, such as compressed air, nitrogen, and atmospheric gases that contact sterile components, containers and closures, and product contact surfaces of processing equipment, such as tanks and piping, must be sterile to prevent contamination of pharmaceuticals and biopharmaceuticals. The sterilization of gases is typically accomplished by passing the gas through one or more sterilizing-grade membrane filters. The filtration process must be designed to ensure that the filtered gas has been sterilized.

GAS VERSUS LIQUID STERILIZING FILTRATION

Sterilizing Filtration of Liquids ⟨1229.4⟩ contains information that is applicable to this chapter, including but not limited to sterilizing-grade filters, retention mechanisms and factors affecting retention, validation, integrity-testing principles and methods, and prefiltration bioburden control. The significant differences between liquid and gas filtration will be discussed in this chapter.

MEMBRANES FOR GAS STERILIZATION

Filters used for sterilization of gases are commonly hydrophobic membranes, although hydrophilic membranes can be employed where membrane wetting during use is not an issue. Wetting may be a factor with filters used for autoclave venting and where vent filters are required to maintain the sterility of tank headspace. Microporous filter membranes that have a pore-size rating of NMT 0.2 µm, which meets the ASTM International standard (see F838-15a *Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration*), are suitable for the sterilization of gases; in fact, these filters are considerably more retentive for gases than for liquids (*1*). Additionally, the filters must be chemically compatible with the gas to be filtered and physically compatible with the filtration conditions (e.g., pressure differentials and flow rates).

RETENTION MECHANISMS

The primary retention mechanisms of membrane filters used in gas sterilization are size exclusion, impaction, diffusional interception, and electrostatic attraction. Size exclusion is effective for particles (microorganisms) that are too large to pass through the pore structure of the filter membrane and is essentially independent of the velocity of gas flowing through the filter. Retention mechanisms other than size exclusion can be effective for particles smaller than the pores they encounter. Retention due to impaction, diffusional interception, and electrostatic attraction is dependent on particle size, flow rate, and relative humidity. For example, particle retention efficiency due to impaction is proportional to the mass of the particle; heavier particles have a greater momentum than lighter ones and are therefore more likely to contact pore walls and subsequently be retained by the filter during tortuous flow. In contrast, the capture efficiency of diffusional interception is inversely proportional to particle size and velocity. Smaller particles influenced by Brownian motion can exit the streamlines, contact the filter matrix, and be retained, and lower velocity increases potential contact time and improves capture efficiency. Electrostatic attraction of particles is reduced by a high relative humidity in the filtered gas. Adsorption, which can be important in liquid filtration, is not a retention mechanism for gases.

VALIDATION

Validation of the processes used in sterilizing filtration for gases, in general, may be divided into two parts: sterilization of the filter and its housing, and the ability of the filter to remove microorganisms from the filtered gas.

Sterilization of the filter and its housing has been addressed specifically in ⟨1229.4⟩ and *Sterilization-in-Place* ⟨1229.13⟩, and generally in *Sterilization of Compendial Articles* ⟨1229⟩.

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Membrane filters meeting the requirements of ASTM F838-15a are suitable for sterilizing filtration of gases in terms of microbial retention. Liquid bacterial challenge testing represents a worst-case condition for sterilizing gas filters because the retention efficiency in liquids is lower than in gases (*2*).

INTEGRITY TESTING

Integrity testing requirements and techniques are discussed in ⟨1229.4⟩. The hydrophobic filters typically used for gas filtration can be integrity tested using bubble-point, diffusive-flow, and pressure-hold tests that use a liquid of low surface tension to completely wet the pore structure of the filter membrane. Additionally, integrity testing can be performed by measuring the membrane's resistance to wetting with water as a function of pressure. Water intrusion testing is an example of this type of integrity testing.

REFERENCES

1. Liu BYH, Rubow KL, Pui DYH. Performance of HEPA and ULPA filters. Proceedings, Annual Technical Meeting—Institute of Environmental Sciences. 1985 Apr 29–May 2; Las Vegas, NV.
2. Parenteral Drug Association. Sterilizing filtration of gases. Technical Report No. 40. *PDA J Pharm Sci Technol.* 2005;58(1 Suppl TR40):7–44.

Add the following:

▲⟨1229.16⟩ PRION INACTIVATION

INTRODUCTION

Prions are transmissible agents comprised of abnormal proteinaceous materials that lead to the precipitation of healthy protein within eukaryotic cells, leading to cell death. Prions have been associated with transmissible spongiform encephalopathies (TSE), fatal neurological diseases, that occur in humans and other animals. Some examples of TSE are Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. Prions are considered highly resistant to most physical and chemical destruction agents, and in many cases are more resistant than bacterial spores to these agents (*1*). Prions are potential contaminants in materials and cell lines of mammalian origin and their inactivation/removal is required where their presence can be expected. They can also be found on equipment and medical devices exposed to contaminated materials. Empirical confirmation is recommended in the application of all prion inactivation processes as there are different contaminants, conflicting statements regarding performance, and limited information on many of the potential processes.

The removal of proteinaceous materials including prions is made more difficult if these are allowed to dry on surfaces before treatment. For that reason all surfaces should be kept water wet until inactivation begins.

The process conditions and agents used for prion inactivation must consider patient safety as part of the risk assessment during product manufacture and reprocessing of equipment and medical devices. The effect of the process/agent on the materials being processed must be considered.

When the items/materials to be treated are intended for disposal, incineration is considered to be the most effective means of prion inactivation.

METHODS OF DESTRUCTION

Chemical Methods

Physical removal and prion degradation (or fragmentation) have been demonstrated using a variety of alkaline cleaning formulations, typically at pH >9. The usefulness of these harsh solutions varies considerably depending on the formulation, concentration, pH, and cleaning process conditions (e.g., exposure time, temperature). Reported results regarding the chemical inactivation of prions have been inconsistent because of numerous variables involving the prion (e.g., strain, concentration, underlying tissue, test species) and the chemical treatment (e.g., agent, concentration, process parameters). Effective prion-inactivating chemical agents include chlorine (delivered in various forms), phenolic compounds, guanidine thiocyanate, and sodium hydroxide, among others (*2*). Chemical methods (with or without heat) are better suited for surface treatment.

Thermal Methods

The destruction of prions by moist heat has been reported across a wide range of temperatures (from 100°/15 min to 138°/1 h), which reflects the diversity of the challenge presented (*2*). Thermal treatments can be used for raw materials, fluids, and equipment.

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Combination Methods

The combination of chemical and thermal methods provides the greatest confidence in prion titer reduction for equipment. A common process for prion decontamination is moist heat (or superheating under pressure) over time (e.g., 121° for ≥1 h, 132°–136° for ≥18 min) immersed in concentrated alkaline solutions such as sodium hydroxide (NaOH) at 1–2 N. Lower concentrations of sodium hydroxide and potassium hydroxide have been shown to be effective against surface prion contamination when used in combination with surfactants supplemented with other chemical additives. An example is a disinfectant formulation containing 0.2% sodium dodecyl sulfate (SDS), 0.3% sodium hydroxide, and 20% *n*-propanol; this combination has been shown to be effective against prions, bacteria, and viruses. The effects of alkali and steam sterilization on prions have been studied and shown to cause protein and peptide fragmentation over time; these effects can be enhanced at increased concentrations of the alkali solution and/or higher temperatures (*1*). When working with hazardous chemical methods, there are practical problems that need to be considered, such as risks to personnel while handling harsh chemical agents, the eventual deleterious effects on the autoclave chamber, and the potential detrimental effects on medical devices due to surface reactive damage to metals and plastics. In addition to sodium hydroxide, sodium hypochlorite (chlorine) solutions are widely used as anti-prion chemicals (*3*).

METHODS THAT ARE NOT EFFECTIVE

The following disinfectants or disinfection processes have been shown to be ineffective for prion decontamination: formaldehyde, glutaraldehyde, alcohols, radiation (ionizing or nonionizing), dry heat, ethylene oxide, quaternary ammonium compounds, pasteurization, and boiling (*4*).

REFERENCES

1. Rutala W, Weber D. Creutzfeldt-Jakob disease: Recommendations for disinfection and sterilization. *Clin Infect Dis.* 2001;32(9):1348–1356.
2. McDonnell GE. Mechanisms of microbial resistance. In: *Antisepsis, Disinfection, and Sterilization: Types, Action, and Resistance.* 2nd ed. Sterling, VA:ASM Press;2017:285–393.
3. McDonnell GE. Transmissible spongiform encephalopathies and decontamination. In: Fraise AP, Maillard J, Sattar SA, eds. *Principles and Practice of Disinfection, Preservation, and Sterilization.* 5th ed. Hoboken, NJ:Wiley-Blackwell;2012:208–228.
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7. NIH, Creutzfeldt-Jakob-Disease-Fact-Sheet. https://www.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Fact-Sheets/Creutzfeldt-Jakob-Disease-Fact-Sheet.

▲ (USP 1-Dec-2019)

⟨1230⟩ WATER FOR HEMODIALYSIS APPLICATIONS

GENERAL PURIFICATION CONSIDERATIONS

Chemical and microbial components that can be found in drinking water meeting U.S. Environmental Protection Agency National Primary Drinking Water Regulations (or equivalent) may have the potential to produce significant negative effects in patients undergoing hemodialysis. It is, therefore, necessary to subject the water to further treatment to reduce these components to acceptable levels. The *Water for Hemodialysis* monograph provides bacterial and chemical tests that are required to ensure patient safety. Additional testing is recommended as follows:

1. Excess levels of aluminum, fluorides, and chlorine may be found seasonally in drinking water as a result of chemicals used in water treatment. These components should be monitored in Water for Hemodialysis being produced in accordance with established standard operating procedures. The maximum acceptable levels of these and other elements and compounds, as proposed by AAMI (Association for the Advancement of Medical Instrumentation) are listed in *Table 1*. These attributes should be periodically monitored to ensure they are being controlled by the routine testing performed in accordance with the *Water for Hemodialysis* monograph.
2. A comprehensive validation testing of the system producing Water for Hemodialysis should be performed initially and periodically thereafter to ensure that the water treatment equipment and system sanitization processes are functioning properly.

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Table 1. Maximum Allowable Chemical Levels in Water for Hemodialysis (water used to prepare dialysate and concentrates from powder at a dialysis facility and to reprocess dialyzers for multiple use)*

Element or Compound	Maximum Concentration (mg/L)
Contaminants with documented toxicity in hemodialysis	
Aluminum	0.01
Chloramines	0.1
Free chlorine	0.5
Copper	0.1
Fluoride	0.2
Lead	0.005
Nitrate (as N)	2
Sulfate	100
Zinc	0.1
Contaminants normally included in dialysate	
Calcium	2 (0.1 mEq/L)
Magnesium	4 (0.3 mEq/L)
Potassium	8 (0.2 mEq/L)
Sodium	70 (3.0 mEq/L)
Other contaminants	
Antimony	0.006
Arsenic	0.005
Barium	0.1
Beryllium	0.0004
Cadmium	0.001
Chromium	0.014
Mercury	0.0002
Selenium	0.09
Silver	0.005
Thallium	0.002

* Reprinted with permission from ANSI/AAMI RD62: 2006, "Water treatment equipment for hemodialysis applications", ©Association for the Advancement of Medical Instrumentation, Arlington, VA.

The chemical limits included in *Table 1* have been recognized by federal government agencies as standards for Water for Hemodialysis. Written standard operating procedures for water testing should be established by the physician in charge or the designated facility manager. The test frequency decision should be based upon historical data analysis, the quality of the source water as reported by the municipal water treatment facility or public health agency in the area, etc. Records should be maintained to document levels and any necessary remedial action taken promptly.

Chemical analysis of water components listed should be performed using methods referenced in the American Public Health Association's *Standard Methods for the Examination of Water and Wastewater,* 21st Edition,[1] those referenced in the U.S. Environmental Protection Agency's *Methods for the Determination of Metals in Environmental Samples,*[2] or equivalent methods as referenced in ANSI/AAMI RD 62:2006.

MICROBIAL CONSIDERATIONS

The *Water for Hemodialysis* monograph includes total aerobic microbial count (TAMC) limits of 100 cfu/mL and endotoxin limits of 1 USP Endotoxin Unit/mL. In addition, the absence of *Pseudomonas aeruginosa* should be routinely determined because this is an opportunistic pathogen hazardous to acutely ill hemodialysis patients. Both the high microbial counts and the

[1] American Public Health Association, Washington, DC 20005.
[2] U.S. Environmental Protection Agency Publication EPA-600-R-94-111, Cincinnati, OH.

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presence of *Pseudomonas aeruginosa* can be associated with inadequate water system maintenance and sanitization. Sampling the water should be done at all use points where the water enters the dialysis equipment. Samples should be assayed within 30 minutes of collection or immediately refrigerated and then assayed within 24 hours of collection. The microbial enumeration and absence tests are performed using procedures found in the USP general test chapters *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Quantification of bacterial endotoxins is performed using procedures found in the USP general test chapter *Bacterial Endotoxins Test* ⟨85⟩.

Because of the incubation time required to obtain definitive microbiological results, water systems should be microbiologically monitored to confirm that they continue to produce water of acceptable quality. "Alert" and "Action Levels" are therefore necessary for the monitoring and control of the system. An Alert Level constitutes a warning and does not require a corrective action. An Action Level indicates a drift from normal operating conditions and requires that corrective action be taken to bring the process back into the normal operating range. Exceeding an Alert or Action Level does not imply that water quality has been compromised. The maximum recommended Action Level for a total viable microbial count in the product water should be no greater than 25 cfu/mL, and the maximum recommended Action Level for bacterial endotoxins should be no greater than 0.25 USP Endotoxin Unit/mL. As with all process control values, Action and Alert Levels should be established from normal system monitoring trends and process capabilities in a fashion that allows remedial actions to occur in response to process control level excursions well before specifications are exceeded (also see *Microbial Considerations* under *Water for Pharmaceutical Purposes* ⟨1231⟩).

⟨1231⟩ WATER FOR PHARMACEUTICAL PURPOSES

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1. INTRODUCTION

Water is widely used as a raw material, inactive ingredient, medicinal vehicle, and solvent in the processing, formulation, and manufacture of pharmaceutical products (dosage forms), active pharmaceutical ingredients (APIs), API intermediates, compendial articles, and analytical reagents as well as in cleaning applications.

This is an informational chapter on pharmaceutical water topics and includes some of the chemical and microbiological concerns unique to water and its preparation and uses. The chapter provides information about water quality attributes (that may or may not be included within a water monograph) and processing techniques that can be used to improve water quality. It also discusses water system validation and gives a description of minimum water quality standards that should be considered when selecting a water source including sampling and system controls. It is equally important for water systems to be operated and maintained in a state of control to provide assurance of operational stability and therefore the capability to provide water that meets established water quality standards.

This informational chapter is intended to be educational, and the user should also refer to existing regulations or guidelines that cover U.S. and international [International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) or World Health Organization (WHO)] good manufacturing practice (GMP) issues, as well as operational and engineering guides and/or other regulatory guidance for water [e.g., from the Food and Drug Administration (FDA), Environmental Protection Agency (EPA), or WHO]. This chapter is not, and should not be considered, an all-inclusive document on pharmaceutical waters. It contains basic information and points to be considered for the processing, holding, monitoring, and use of water. It is the user's responsibility to ensure that:

1. The selection of the type and specifications of water is appropriate for its intended use.
2. Water production and quality meet applicable governmental regulations and guidance.
3. The pharmacopeial specifications for the types of water used in monographed articles are met.
4. Water used in the preparation of reagents for analysis or the performance of required tests meets USP requirements.

Control and monitoring of the chemical and endotoxin purity of waters are important for complying with the requirements of the monographs in this compendium. Attributes listed in *USP* monographs should be considered the "minimum" requirements. More stringent requirements may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of waters can be found in the monographs and is also discussed further in this chapter.

Control of the microbiological quality of water is also important for many of its uses. This attribute is intentionally not specified in most water monographs. Microbiological control is discussed throughout this chapter, but especially in sections *4. Validation and Qualification of Water Purification, Storage, and Distribution Systems*, *5. Design and Operation of Purified Water and Water for Injection Systems*, *6. Sampling*, *8. Microbial Evaluations*, and *9. Alert and Action Levels and Specifications*.

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This chapter contains various chemical, microbiological, processing, and engineering concepts of importance to users of water. Water system validation, process control levels, and specifications are also presented later in this chapter.

2. SOURCE WATER CONSIDERATIONS

Source water is the water that enters the facility. The origin of this source water can be from natural surface waters like rivers and reservoirs, deep-bed well waters, sea waters, or some combination of these, potentially including multiple locations of each type of source water. Thus, source water can be supplied from these various origins (public or private), from municipalities' on-site water sourcing, or by external delivery such as a truck. It is possible that source water may not be potable and safe to drink. Such water may require pretreatment to ensure that it meets drinking water standards. It is the responsibility of the users of any source water to ensure that the water used in the production of drug substances (API), as well as water for indirect drug product contact or for purification system feed water purposes meets, at a minimum, drinking (potable) water standards as defined by the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the U.S. EPA or the drinking water regulations of the European Union (EU) or Japan, or the WHO drinking water guidelines (see *3.3.1 Drinking Water*). These regulations establish limits on the types and quantities of certain chemical and microbiological contaminants and ensure that the water will contain safe quantities of chemical and microbial species.

Where water supplies are from regulated water utility companies, less stringent monitoring may be possible because the attributes may be tested regularly and ensured by the supplier (see *9.4.5 Source Water Control*). Water being withdrawn from a nonregulated supply should be sampled and tested appropriately at a suitable frequency that takes into account local environmental and seasonal changes and other quality fluctuations. Testing should ensure conformance with one of the drinking water standards discussed above.

The use of water complying with one of these designated drinking waters as a source water allows water pretreatment systems to only be challenged to remove small quantities of potentially difficult-to-remove chemicals. Control of objectionable chemical contaminants at the source water stage eliminates the need to specifically test for some of them [e.g., trihalomethanes and elemental impurities (see *Elemental Impurities—Limits* ⟨232⟩)] after the water has been further purified, assuming there is no opportunity for recontamination.

Source waters can be used for nonproduct contact purposes such as for non-contact cooling systems. Such water may not normally be required to meet drinking water standards. Under such circumstances, the quality standards for this water when used in a pharmaceutical facility should be subject to quality standards established by the user and defensible to regulatory agencies.

Change to read:

3. WATERS USED FOR PHARMACEUTICAL MANUFACTURING AND TESTING PURPOSES

There are many different grades of water used for pharmaceutical purposes. Several are described in *USP* monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on-site where they are used; and sterile waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of sterile waters that differ in their designated applications, packaging limitations, and other quality attributes. Monographed waters must meet the quality attributes as specified in the related monographs, and any *Notes* appearing in those monographs should be considered and addressed.

With the exception of Bacteriostatic Water for Injection, the monographed bulk and sterile waters have a statement indicating that there are no added substances, or no added antimicrobial agents. In the case of antimicrobial agents, the purpose is to ensure that the sterile water product is rendered sterile based solely on its preparation, packaging, and storage. In the case of the more general statement, "no added substances", this requirement is intended to mean "no added substances that aren't sufficiently removed". Two specific examples support this intention, but there are many examples. First, the use of softeners is commonplace. A softener replaces calcium and magnesium ions ▲(also known as hardness ions)▲ 1S (USP41) with sodium, so technically you are adding two sodium ions for each hard ion. The purpose of sodium displacement is to protect downstream equipment from the hard water. The sodium ions are eventually removed sufficiently, and this is proven when the water sample passes the test in *Water Conductivity* ⟨645⟩. Another specific example is the use of ozone as a sanitant that is added to the storage tank for microbial control. This could be considered an added substance, unless the ozone is destroyed before use, as is normally the case. Other notable examples include the addition of chlorine to kill bacteria in the pretreatment system, use of bisulfite to chemically reduce chlorine to chloride and protect downstream equipment, and use of a nitrogen blanket for protection from atmospheric contamination.

There are also other types of water for which there are no monographs. These are waters with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The descriptive titles may imply certain quality attributes or modes of preparation, but these nonmonographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or specified attributes. Waters produced by other means or controlled by other test attributes, or even a monographed water, may equally satisfy the intended uses for these waters. It is the user's responsibility to ensure that such waters, even if produced and controlled exactly as stated, are suitable for their intended use. Wherever the term "water" is used within this compendium without other descriptive adjectives or clauses, the intent is that water of no less purity than USP *Purified Water* be used (see *3.1.1 Purified Water*). A brief description of the various types of waters commonly associated with pharmaceutical applications and their significant uses or attributes follows.

Figure 1 may be helpful in understanding some of the various types of waters, their preparation, and uses.

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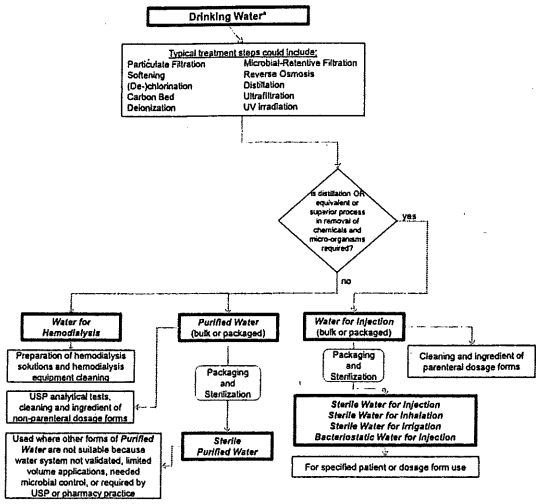

Figure 1. Water for pharmaceutical purposes. [a]Complying with U.S. EPA NPDWR or the drinking water regulations of EU or Japan or WHO.

3.1 Bulk Monographed Waters and Steam

The following waters are generally produced in large volumes using a multiple-unit operation water system. These waters are typically distributed in a piping system for use at the same site.

3.1.1 PURIFIED WATER

Purified Water (see the *USP* monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as the cleaning of nonparenteral product-contact components and equipment. Unless otherwise specified, *Purified Water* is also to be used as the minimum water quality for all tests and assays in which "water" is indicated (see *General Notices, 8.230.30 Water in a Compendial Procedure*). This applies regardless of the font and letter case used in its spelling.

The minimal quality of source water for the production of Purified Water is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified Water must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. Purified Water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified Water systems that function under ambient conditions are particularly susceptible to the establishment of biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the water. These ambient Purified Water systems require frequent sanitization and microbiological monitoring to ensure that the water reaching the points of use has appropriate microbiological quality.

The *Purified Water* monograph also allows bulk packaging for commercial use elsewhere. In contrast to Sterile Purified Water, packaged Purified Water is not required to be sterile. Because there is potential for microbial contamination and other quality changes in this packaged nonsterile water, this form of Purified Water should be prepared and stored in a manner that limits microbial growth, and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

3.1.2 WATER FOR INJECTION

Water for Injection (see the *USP* monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as the cleaning of certain equipment and parenteral product-contact components.

The minimal quality of source water for the production of Water for Injection is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. This source water may be treated to render it suitable for subsequent final purification steps, such as distillation (or whatever other validated process is used, according to the monograph). The finished water must meet all of the chemical requirements specified in the monograph, as well as an additional bacterial endotoxin specification. Because endotoxins are produced by the kinds of microorganisms that are prone to inhabit water systems, the equipment and procedures used by the system to purify, store, and distribute Water for Injection should be designed to control microbial contamination and must be designed to remove incoming endotoxins from the source water. Water for Injection systems must be validated to reliably and consistently produce and distribute this quality of water.

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The *Water for Injection* monograph also allows bulk packaging for commercial use. In contrast to Sterile Water for Injection, packaged Water for Injection is not required to be sterile. However, to preclude significant changes in its microbial and endotoxins content during storage, this form of Water for Injection should be prepared and stored in a manner that limits microbial introduction and growth and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

3.1.3 WATER FOR HEMODIALYSIS

Water for Hemodialysis (see the *USP* monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. The minimal quality of source water for the production of Water for Hemodialysis is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. Water for Hemodialysis has been further purified to reduce chemical and microbiological components, and it is produced and used on site. This water contains no added antimicrobial agents, and it is not intended for injection. Water for Hemodialysis must meet all of the chemical requirements specified in the monograph as well as an additional bacterial endotoxin specification. The microbial limits attribute for this water is unique among the "bulk" water monographs, but is justified on the basis of this water's specific application, which has microbial content requirements related to its safe use. The bacterial endotoxins attribute is likewise established at a level related to its safe use.

3.1.4 PURE STEAM

Pure Steam (see the *USP* monograph) is also sometimes referred to as "clean steam". It is used where the steam or its condensate would directly contact official articles or article-contact surfaces, such as during their preparation, sterilization, or cleaning where no subsequent processing step is used to remove any impurity residues. These Pure Steam applications include, but are not limited to, porous load sterilization processes, product or cleaning solutions heated by direct steam injection, or humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

The minimal quality of source water for the production of Pure Steam is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO, and which has been suitably treated. The water is then vaporized with suitable mist elimination, and distributed under pressure. The sources of undesirable contaminants within Pure Steam could arise from entrained source water droplets, anticorrosion steam additives, or residues from the steam production and distribution system itself. The chemical tests in the *Pure Steam* monograph should detect most of the contaminants that could arise from these sources. If an official article is exposed to Pure Steam and it is intended for parenteral use or other applications where the pyrogenic content must be controlled, the Pure Steam must additionally meet the specification for *Bacterial Endotoxins Test* ⟨85⟩.

These purity attributes are measured in the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the process for Pure Steam condensate generation and collection, because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, particularly the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, may also be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam's sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen because the steam is extremely hot and is in a persistent superheated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces would thereby be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, may also be important for certain Pure Steam applications. However, because these additional attributes are use-specific, they are not mentioned in the *Pure Steam* monograph.

Note that lower-purity "plant steam" may be used in the following applications: 1) for steam sterilization of nonproduct-contact nonporous loads, 2) for general cleaning of nonproduct-contact equipment, 3) as a nonproduct-contact heat-exchange medium, and 4) in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

Finally, because Pure Steam is lethal to microbes, monitoring of microbial control within a steam system is unnecessary, as is microbial analysis of the steam condensate.

3.2 Sterile Monographed Waters

The following monographed waters are packaged forms of either Purified Water or Water for Injection that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names, and may also have restrictions on the packaging configurations related to those uses. In general, these sterile waters may be used in a variety of applications in lieu of the bulk forms of water from which they were derived. However, there is a substantial difference between the acceptance criteria for the chemical purities of these bulk waters versus sterile waters. The specifications for sterile waters differ from those of bulk waters to accommodate a wide variety of packaging types, properties, volumes, and uses. As a result, the inorganic and organic impurity specifications are not equivalent for bulk and packaged waters. The packaging materials and elastomeric closures are the primary sources of these impurities, which tend to increase over the shelf life of these packaged articles. Therefore, due consideration must be given to the chemical purity suitability at the time of use of the sterile forms of water when used in manufacturing, analytical, and cleaning applications in lieu of the bulk waters from

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which these waters were derived. It is the user's responsibility to ensure fitness for use of these sterile packaged waters in these applications. Nevertheless, for the applications discussed below for each sterile water, their respective purities and packaging restrictions generally render them suitable by definition.

3.2.1 STERILE PURIFIED WATER

Sterile Purified Water (see the *USP* monograph) is Purified Water, packaged and rendered sterile. It can be used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring Purified Water where 1) access to a validated Purified Water system is not practical, 2) only a relatively small quantity is needed, 3) Sterile Purified Water is required by specific monograph or pharmacy practice, or 4) bulk packaged Purified Water is not suitably controlled for the microbiological quality for its intended use.

3.2.2 STERILE WATER FOR INJECTION

Sterile Water for Injection (see the *USP* monograph) is Water for Injection packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. It may also be used for other applications where bulk Water for Injection or Purified Water is indicated but access to a validated water system is not practical, or where only a relatively small quantity is needed. Sterile Water for Injection is packaged in single-dose containers not larger than 1 L.

3.2.3 BACTERIOSTATIC WATER FOR INJECTION

Bacteriostatic Water for Injection (see the *USP* monograph) is Water for Injection, packaged and rendered sterile, to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

3.2.4 STERILE WATER FOR IRRIGATION

Sterile Water for Irrigation (see the *USP* monograph) is Water for Injection packaged and sterilized in single-dose containers that may be larger than 1 L and allow rapid delivery of their contents. Due to its usage, Sterile Water for Irrigation is not required to meet *Particulate Matter in Injections* ⟨788⟩. It may also be used in other applications that do not have particulate matter specifications, where bulk Water for Injection or Purified Water is indicated but where access to a validated water system is not practical, or where somewhat larger quantities are needed than are provided as Sterile Water for Injection.

3.2.5 STERILE WATER FOR INHALATION

Sterile Water for Inhalation (see the *USP* monograph) is Water for Injection that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. This monograph has no requirement to meet ⟨788⟩; it carries a less stringent specification for bacterial endotoxins than Sterile Water for Injection, and therefore is not suitable for parenteral applications.

3.3 Nonmonographed Waters

In addition to the bulk monographed waters described above, nonmonographed waters can also be used in pharmaceutical processing steps such as cleaning and synthetic steps, and also as a starting material for further purification or testing purposes. Unless otherwise specified in the compendium, the minimum quality of water is *Purified Water*. [NOTE—The information in this chapter is not an all-inclusive discussion of all nonmonographed waters identified in the *USP–NF*.]

3.3.1 DRINKING WATER

Drinking Water can be referred to as Potable Water (meaning drinkable or fit to drink), National Primary Drinking Water, Primary Drinking Water, or EPA Drinking Water. Except where a singular drinking water specification is stated (such as the U.S. EPA's NPDWR, as cited in 40 CFR Part 141), this water must comply with the quality attributes of either the NPDWR or the drinking water regulations of the EU or Japan, or the *WHO Guidelines for Drinking-Water Quality*. Drinking Water may originate from a variety of sources including a public water supply, a private water supply (e.g., a well), or a combination of these sources (see *2. Source Water Considerations*).

Drinking Water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking Water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the contaminant levels allowed in Drinking Water are generally considered safe for use in preparing official substances and other drug substances. However, where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even water as pure as Water for Injection or Purified Water. Such higher-purity waters, however, might require only selected attributes to be of higher purity than Drinking Water (see *Figure 2a* and *Figure 2b*). Drinking Water is the prescribed source or feed water for the production of bulk monographed pharmaceutical waters. The use of Drinking Water specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. Because seasonal variations in the quality attributes of the Drinking Water supply can occur, it is important to give due consideration to its uses. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.

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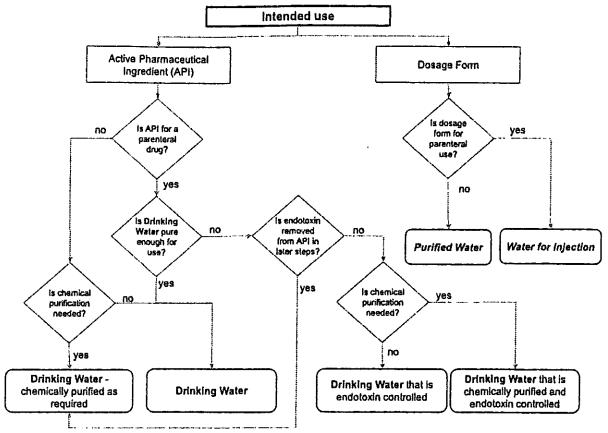

Figure 2a. Selection of water for pharmaceutical purposes: APIs and dosage forms.

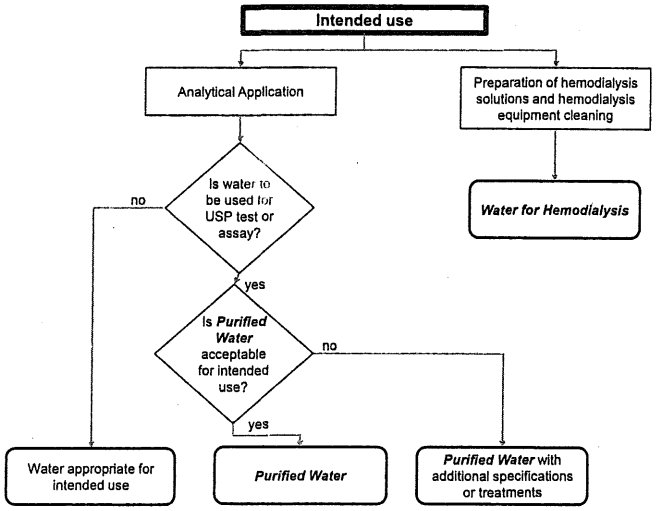

Figure 2b. Selection of water for pharmaceutical purposes: Analytical reagents.

3.3.2 OTHER NONMONOGRAPHED WATERS

In addition to Drinking Water, this compendium discusses waters with various other designations. These include waters of various quality levels for special uses such as, but not limited to, cleaning and testing purposes.

Both *General Notices and Requirements* (see *General Notices, 8.230.30 Water in a Compendial Procedure*) and *Reagents, Indicators, and Solutions* clearly state that where the term "water" is indicated for use in analyses without grammatical qualification or other specification, the quality of the water must be *Purified Water*. However, numerous such qualifications do exist. Some of these qualifications involve adjectives describing methods of preparation, ranging from specifying the primary purification step to specifying additional purification. Other qualifications call for specific attribute "absences" to be met that might otherwise interfere with analytical processes. In most of these cases, the required attribute absences are not specifically tested. Sometimes, a further "purification process" is specified that ostensibly allows the water to adequately meet this required "absence attribute".

However, preparation instructions for many reagents were carried forward from the innovator's laboratories to the originally introduced monograph for a particular *USP–NF* article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator's laboratory. These specific water designations may have originated without the innovator's awareness of the requirement for *Purified Water* in *USP–NF* tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters could now be met by the basic preparation steps and current specifications of *Purified Water*. In some cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to utilize specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability of operations for producing these alternative analytical waters should be verified so that the desired attributes are produced. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. The following is a summary of the various types of nonmonographed analytical waters that are cited in the *USP–NF*. This is not an exhaustive listing. Those listed below are used in multiple locations. Several nonmonographed analytical waters are not included below because they are only found in one or perhaps two locations within this compendium.

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Note that the names of many of the waters below imply a very low chemical impurity level. For example, "deionized water" implies that all the ions have been removed. However, in most cases discussed below, exposure of the water to air will result in the ingress of carbon dioxide (CO_2), leading to the formation of bicarbonate and hydrogen ions. Therefore, the removal of ions cannot be completely maintained for most analytical applications.

3.3.3 AMMONIA-FREE WATER

From a functional standpoint, Ammonia-Free Water must have a negligible ammonia concentration to avoid interference in tests sensitive for or to ammonia. Due to the nature of the uses of this water, *Purified Water* could be a reasonable alternative for these applications.

3.3.4 CARBON DIOXIDE-FREE WATER

Carbon dioxide-free water is defined in the *Reagents, Indicators, and Solutions* section of *USP–NF* as Purified Water that has been vigorously boiled for NLT 5 min, then cooled and protected from absorption of atmospheric carbon dioxide. Alternatively, this could be *Purified Water* that has a resistivity of NLT 18 megohm-cm at 25°.

Because the absorption of atmospheric carbon dioxide lowers the pH of high-purity waters, most of the uses of Carbon Dioxide-Free Water are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in bicarbonate-sensitive reagents or determinations.

The term "Carbon Dioxide-Free Water" is sometimes used improperly. Besides its use for pH or acidity/alkalinity tests, the purpose for using this water is not always clear. The intention could be to use water that was deaerated (free of dissolved air) or deionized (free of extraneous ions), or even Purified Water with an additional boiling step. Although boiling is highly effective for removing carbon dioxide as well as all other dissolved gasses, these gases are readily re-absorbed unless the water is protected. Even with protection, such as use of a stoppered container, re-absorption will occur over time as air will readily transmit through seals and diffuse through most materials. Deionization is also an efficient process for removing dissolved carbon dioxide. Carbon dioxide forms ionic bicarbonate in water, and will be subsequently removed by ion-exchange resins. However, the same problem of carbon dioxide re-absorption will occur after the deionized water is exposed to air. Also, the deionization approach for creating Carbon Dioxide-Free Water does not deaerate the water or remove other dissolved gases such as oxygen (O_2); it only removes carbon dioxide and other ions.

Depending on the application, *Purified Water* may meet the requirements where Carbon Dioxide-Free Water is called for. This could also include pH or acidity or alkalinity tests. The pH of a sample of pure Deionized Water is, by definition, 7.0. When that same sample is exposed to typical environmental atmospheric conditions, the water sample will absorb carbon dioxide and result in a pH range of approximately 5.4–6.2 ($[H^+]$ is in the range of 4.0×10^{-6} M to 6.3×10^{-7} M). The added acidity caused by carbon dioxide absorption may be insignificant compared to the material being analyzed.

3.3.5 DISTILLED WATER

Distilled Water is produced by vaporizing Drinking Water or a higher quality of water and condensing it into a purer state. It is used primarily as a solvent for reagent preparation, and it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. Distilled Water is also cited as the starting water to be used for making High-Purity Water (see *3.3.10 High-Purity Water*). Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for *Purified Water* derived by other means of purification or *Water for Injection* could be equally suitable where Distilled Water is specified. It is the user's responsibility to verify the suitability of Purified Water or Water for Injection.

3.3.6 FRESHLY DISTILLED WATER

Freshly Distilled Water or "recently distilled water" is produced in the same manner as Distilled Water and should be used soon after its generation. This implies the need to avoid endotoxin contamination, as well as any other forms of contamination from the air or containers, that could arise with prolonged storage. Freshly Distilled Water is used for preparing solutions for subcutaneous test-animal injections and for a reagent solvent in tests for which there appears to be no particularly high water purity needed that could be ascribable to being "freshly distilled". In the test-animal application, the term "freshly distilled" and its testing use imply a chemical, endotoxin, and microbiological purity that could be equally satisfied by *Water for Injection* (although no reference is made to these chemical, endotoxin, or microbial attributes or specific protection from recontamination). For non-animal uses, water meeting the requirements for *Purified Water* derived by other means of purification and/or storage periods could be equally suitable where "recently distilled water" or Freshly Distilled Water is specified. It is the user's responsibility to verify the suitability of Purified Water or Water for Injection.

3.3.7 DEIONIZED WATER

Deionized Water can be produced by starting with either Drinking Water or Purified Water, depending upon monograph or testing procedures defined in the compendia. Deionized Water is produced by an ion-exchange process in which the cations and anions are replaced with H+ and OH– ions by use of ion-exchange resins. Similar to Distilled Water, Deionized Water is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for *Purified Water* that is derived by other means of purification could be equally suitable where Deionized Water is specified. It is the user's responsibility to verify the suitability of Purified Water.

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3.3.8 DEIONIZED DISTILLED WATER

Deionized Distilled Water is produced by deionizing (see *3.3.7 Deionized Water*) Distilled Water. This water is used as a reagent in a liquid chromatography test that requires a low ionic or organic impurity level. Because of the importance of this high purity, water that meets the requirements for *Purified Water* may not be acceptable. High-Purity Water (see *3.3.10 High-Purity Water*) could be a reasonable alternative to this water. It is the user's responsibility to verify the suitability of the alternative water used.

3.3.9 FILTERED WATER

Filtered Water is Purified Water that has been filtered to remove particles that could interfere with the analysis where this water is specified. It is sometimes used synonymously with Particle-Free Water and Ultra-Filtered Water and is cited in some monographs and general chapters as well as in *Reagents, Indicators, and Solutions*. Depending on its referenced location in *USP–NF*, it is variously defined as water that has been passed through filters rated as 1.2, 0.2, or 0.22 µm, or unspecified porosity rating. Even though the water names and the filter ratings used to produce these waters are defined inconsistently, the use of 0.2-µm or 0.22-µm filtered *Purified Water* should be universally acceptable for all applications where Particle-Free Water, Filtered Water, or Ultra-Filtered Water are specified.

3.3.10 HIGH-PURITY WATER

High-Purity Water may be prepared by deionizing previously distilled water and then filtering it through a 0.45-µm rated membrane. This water must have an in-line conductivity of NMT 0.15 µS/cm (NLT 6.67 megohm-cm) at 25°. If the water of this purity contacts the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately increase by as much as about 1.0 µS/cm ▲at 25°▲ 1S (USP41) as atmospheric carbon dioxide dissolves in the water and equilibrates to hydrogen and bicarbonate ions. Therefore, if the analytical use requires that water conductivity remains as low as possible or the bicarbonate/carbon dioxide levels be as low as possible, the water should be protected from atmospheric exposure. High-Purity Water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning where less stringent water specifications would not be considered acceptable. However, if a user's routinely available *Purified Water* is filtered and meets or exceeds the conductivity specifications of High-Purity Water, it could be used in lieu of High-Purity Water.

3.3.11 DEAERATED WATER

Deaerated Water or "degassed water" is Purified Water that has been treated to reduce the content of dissolved air by "suitable means" such as boiling, sonication, and/or stirring during the application of a partial vacuum, followed by immediate use or protection from air reabsorption.

3.3.12 OXYGEN-FREE WATER

Oxygen-Free Water is Purified Water that has been treated to remove or reduce dissolved oxygen. Such treatment could involve deaerating by boiling or sparging with an inert gas such as nitrogen or helium, followed by inert gas blanketing to prevent oxygen reabsorption. Any procedure used for removing oxygen should be verified as reliably producing water that is fit for use.

3.3.13 WATER FOR BACTERIAL ENDOTOXINS TEST

Water for Bacterial Endotoxins Test (BET) is also referred to as Limulus Amebocyte Lysate (LAL) Reagent Water. This type of water is often Water for Injection, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the LAL reagent used in the BET (see ⟨85⟩).

Change to read:

4. VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

4.1 Validation Requirement

Establishing the reliability of pharmaceutical water purification, storage, and distribution systems requires demonstrating control of the process through an appropriate period of monitoring and observation. Finished water is typically continuously produced and used, while product and process attributes may only be periodically assessed. The quality of bulk finished water cannot be established by only testing monograph attributes. The unit operations in the pharmaceutical water system need to demonstrate that they are in control through monitoring of the process parameters and water quality. The advent of using conductivity and total organic carbon (TOC) to define chemical purity allows the user to more quantitatively assess the water's chemical purity and its variability as a function of routine treatment system maintenance and regeneration. Treatment processes must also demonstrate control of microbial attributes within the overall system. Some unit operations that are needed for chemical treatment may significantly increase microbial and bacterial endotoxin levels. These are later controlled by downstream unit operations. Knowledge of the treatment system processes and the effectiveness of control measures is needed to ensure that the pharmaceutical waters are acceptable for use.

Efficacy of the design, operation, sanitization, and control of the pharmaceutical water system is demonstrated through the monitoring of chemical and microbial attributes. A typical water system validation program involves an initial increased

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frequency of monitoring of the treatment system process parameters and sampling and testing of major process points to demonstrate the ability to produce the acceptable water and to characterize the operation of the system. This is followed by a life cycle approach of validation maintenance and monitoring.

4.2 Validation Approach

Validation is the program of documenting, to a high level of assurance, that a specific process is capable of consistently delivering product conforming to an established set of quality attributes. A validation program qualifies and documents the design, installation, operation, and performance of the system. A graphical representation of a typical water system validation life cycle is shown in *Figure 3*.

The validation protocol should be based on the boundaries of the water system and the critical water quality and process attributes needed to maintain consistent performance. The system boundary may stop at the point of use or may include the water transfer process. If the transfer process from the distribution system outlets to the water use locations (typically either with hoses or hard-piped equipment connections) is defined as outside the water system boundary, then this transfer process still needs to be validated to not adversely affect the quality of the water as it is delivered for use. Because routine quality control (QC) microbial monitoring is performed for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see *6.1.2 QC Sampling*), there is some logic to include this water transfer process within the distribution system validation.

4.2.1 VALIDATION ELEMENTS

Validation is accomplished through the use of a structured, documented process. The phases of this process include Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), Performance Qualification (PQ), and Validation Maintenance. The process is documented in a validation protocol. The elements may be in individual protocols for each phase, or integrated into variations of a DQ/IQ/OQ/PQ combined document format. The protocols are formally approved quality documents. Factory Acceptance Testing (FAT), Site Acceptance Testing (SAT), and commissioning testing of the system may supplement qualification tests for IQ or OQ provided that they are properly documented and reviewed; and if it can be shown that the system functionality is not affected by the transport and installation.

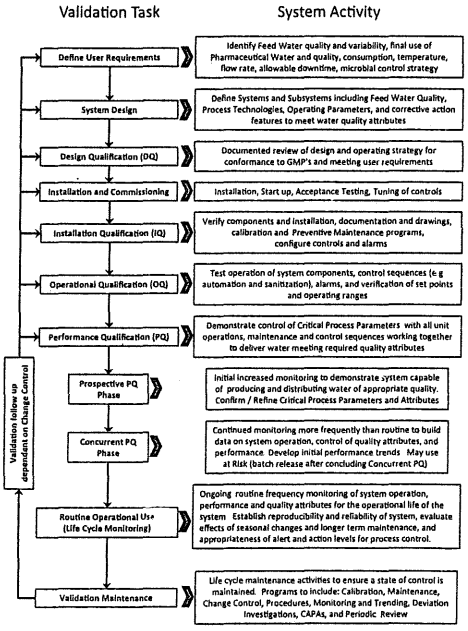

Figure 3. Water system validation life cycle.

4.2.2 USER REQUIREMENTS SPECIFICATION AND DESIGN QUALIFICATION

The user requirements for the water system should identify the design, operation, maintenance, and quality elements needed to produce the desired water type from the available source water, including its anticipated attribute variability. The essential elements of quality need to be built in at this stage and any GMP risks mitigated to an acceptable level.

The review of the specifications, system design, components, functions, and operation should be performed to demonstrate that the system complies with GMPs and verify that the design meets the user requirements. This documented review may be performed as part of the overall design process or as a separate DQ.

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4.2.3 IQ

An IQ protocol for a water system confirms that the system has been properly installed and documented. This may include verification of components, piping, installation, and weld quality; documentation of the specifications for all system components present; inspections to verify that the drawings accurately depict the final configuration of the water system and, where necessary, special tests to verify that the installation meets the design requirements. Additionally, the water system is readied for operational testing, including calibration of instruments, configuration of alarm levels and adjustment of operating parameters (e.g., flow rate, pressure).

4.2.4 OQ

The OQ phase consisting of tests and inspections to verify that the equipment, system alerts, and controls are operating reliably and that appropriate Alert and Action Levels are established (this phase of qualification may overlap with aspects of IQ and PQ). During this phase of validation specific testing is performed for alarms, verifying control sequences, equipment functional checks, and verification of operating ranges. SOPs for all aspects of water system operation, maintenance, water use, water sampling, and testing, etc. should be in place and operator training completed. At the completion of the OQ, the water system has demonstrated that the components are operational and the system is producing suitable water.

4.2.5 PQ

The prospective PQ stage considers two aspects of the water system: critical process parameters and critical water attribute parameters. These are evaluated in parallel by monitoring the water quality and demonstrating acceptable quality attributes while demonstrating control of the process parameters (see *6.3 Validation Sampling Plans*). The initial PQ stage may result in refinement of process parameters to yield appropriate water quality. This PQ stage includes an increased frequency of monitoring for approximately 2–4 weeks, or sufficient time to generate adequate data to demonstrate that water meeting the appropriate quality attributes is produced and distributed. One of the reasons for this duration is that biofilm, the source of planktonic organisms in water samples, takes time to develop and to determine if the sanitization unit operations and processes are adequate to control microbial proliferation. The chemical control program adequacy is typically apparent in less time than it takes to see microbial control adequacy. However, chemical purification can be compromised by poor microbial control and, to a lesser degree, vice versa.

Once a level of control of microbial and chemical attributes has been demonstrated, the next phase of PQ is to continue the frequency of monitoring for approximately 2–4 weeks at a somewhat reduced level that will still give adequate data on system performance while using the pharmaceutical water. The water may be used for manufacturing at risk, and the associated products may be released only after water quality attributes have been determined to be acceptable and this validation phase has been completed. At the completion of the second phase, the data should be formally reviewed and the system approved for operational use.

4.3 Operational Use

When the water system has been placed into operational use, monitoring of the water quality attributes and the system process parameters is performed at a routine frequency (see *6.4 Routine Sampling Plans*) to ensure that they remain with a state of control during long-term variability from seasonal variations in source water quality, unit operation maintenance, system sanitization processes, and earlier-established Alert and Action Levels.

The water system should continue to be monitored and evaluated on an on-going basis following a life cycle approach using online instruments or samples for laboratory-based testing. The use of online instruments and process automation technology, such as conductivity, TOC, temperature, flow rate, and pressure can facilitate improved operational control of the attributes and parameters and for process release. Manual observation of operating parameters and laboratory-based testing is also appropriate and acceptable for monitoring and trend evaluation.

4.3.1 MONITORING

The frequency of routine monitoring should be based on the criticality of the finished water, capabilities of the process, and ability to maintain product water quality trends. Monitoring may be adjusted from the initial validation monitoring program when there is sufficient data to support a change (see *6.4 Routine Sampling Plans*).

4.3.2 VALIDATION MAINTENANCE

Maintaining the validated state of control requires a life cycle approach. After the completion of the PQ and release of the water system for use, ongoing activities and programs have to be in place to maintain the validated state of control after the system has been validated and placed into service (see *5.4 Operation, Maintenance, and Control*). This includes unit operation, calibration, corrective maintenance, preventive maintenance, procedures, manuals and drawings, standardization of instruments, process parameter and quality attribute trending, change control, deviations, corrective and preventive actions (CAPA), training, records retention, logbooks, etc.

4.3.3 CHANGE CONTROL

Identification and control of changes made to unit operations and other system components, operation parameters, system sanitization, and laboratory processes or procedures need to be established. Not all changes will require validation follow up, but even minor ones, such as gasket elastomer changes could have an impact on quality attributes. The impact of the change

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on process parameters and quality attributes must be identified, evaluated and remediated. This may result in a selective validation activity to demonstrate the ongoing state of control for the system and ability to maintain water quality attributes.

Certain calibration and preventive maintenance activities may be considered routine tasks if they do not impact on system operation or water quality. Replacement of components needs to be carefully evaluated. Replacement of components using exact parts generally does not affect system operation or control. Replacement of components with ones that ▲are not exact parts but have▲ 1s (USP41) similar functional specifications can be performed at risk with the critical specifications (e.g., material of construction, dimensions, flow rate, response factors) having been evaluated and the differences determined to be acceptable and documented within the change control system.

4.3.4 PERIODIC REVIEW

The water system qualification, maintenance history, calibration records, quality and process data, issues with the unit operations and any process variability, change control, and other validation maintenance data should be assessed periodically to determine impact on the state of control.

The review may result in adjustments to operating or sanitization processes, calibration or maintenance plans, or monitoring plans. This may also result in additional testing or repeating certain qualification tasks (re-qualification).

Change to read:

5. DESIGN AND OPERATION OF PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce Purified Water and Water for Injection include similar components, control techniques, and procedures. The quality attributes of the two waters differ in their bioburden expectation, the presence of a bacterial endotoxin requirement for Water for Injection, and in their methods of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure removal of bacteria and bacterial endotoxins and reductions in opportunities for biofilm re-development within those purification steps that could become in situ sources of bacteria and endotoxin in the finished water.

Many aspects of system design and operation relate to control and elimination of biofilm. Unit operations can cause the deterioration of water microbial attributes and the formation of biofilm on unit operation surfaces, even when properly maintained (see *8.2 Biofilm Formation in Water Systems*).

Production of pharmaceutical water involves sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process for selecting an appropriate water quality for a particular pharmaceutical purpose is shown in the decision trees in *Figure 2a* and *Figure 2b*. This diagram may be used to assist in defining requirements for specific water uses and in the selection of unit operations. The final unit operation used to produce Water for Injection is limited to distillation or other processes equivalent or superior to distillation in the removal of chemical impurities as well as microorganisms and their components, such as bacterial endotoxins. Distillation coupled with suitable pretreatment technologies has a long history of generally reliable performance (though not completely infallible) and can be validated as a unit operation for the production of Water for Injection. Other combinations of purification technologies may also be suitable in the production of Water for Injection if they can be shown through validation to be as effective and reliable as distillation in the removal of chemicals and microorganisms. The development of new designs and materials of construction for other technologies (such as reverse osmosis, electrodeionization, and ultrafiltration) that allow intermittent or continuous operation at hot bactericidal conditions show promise for a valid use in producing Water for Injection.

5.1 Unit Operations Considerations

To achieve the quality attributes for pharmaceutical waters, multiple-unit operations are required. The design of the water purification system needs to take into consideration different aspects, including the source water quality, sanitization, pharmaceutical water quality attributes, uses of the water, and maintenance programs. Each unit operation contributes specific purification attributes associated with chemical and microbiological parameters.

The following is a brief description of selected unit operations and the design, installation, operation, maintenance, and monitoring parameter considerations associated with them. Not all unit operations are discussed, nor are all potential shortcomings addressed.

5.1.1 PREFILTRATION

The purpose of prefiltration—also referred to as initial, coarse, particulate, or depth filtration—is to remove solid contaminants from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology primarily uses sieving effects for particle capture and a depth of filtration medium that has a high "dirt load" capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in the type of filtering media and the location in the process. Granular or cartridge prefilters are often situated at the beginning of the water purification system prior to unit operations designed to remove the source water disinfectants. Cartridge-type coarse filters may also be used to capture fines released from granular beds such as activated carbon and deionization beds. These locations, however, do not preclude the need for periodic microbial evaluation.

Design and operational issues that may impact the performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control methods involve pressure

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and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing of the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

5.1.2 ACTIVATED CARBON

Activated carbon beds, depending on the type and placement, are used to adsorb low-molecular-weight organic material, bacterial endotoxins, and oxidizing additives such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reactions with downstream unit operations, stainless steel surfaces, resins, and membranes.

The chief operating concerns regarding activated carbon beds include the propensity to support bacterial growth, the potential for hydraulic channeling, the organic adsorption capacity, and insufficient contact time. Operation deficiencies may result in the release of bacteria, endotoxins, organic chemicals, and fine carbon particles.

Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. Monitoring of carbon bed unit operation may also include microbial loading, disinfectant chemical reduction, and TOC if used for TOC reduction. The use of hot water or steam for carbon bed sanitization is ineffective if there is channeling rather than even permeation through the bed. Channeling can be mitigated through design and proper flow rates during sanitization.

Microbial biofilm development on the surface of the granular carbon particles can cause adjacent bed granules to agglomerate. This may result in ineffective removal of trapped debris and fragile biofilm during backwashing, and ineffective sanitization.

Alternative technologies to activated carbon beds can be used to avoid their microbial challenges. These include disinfectant-neutralizing chemical additives and intense ultraviolet (UV) light for removal of chlorine, and regenerable organic scavenging deionizing resins for removal of organics.

5.1.3 ADDITIVES

Chemical additives are used in water systems 1) to control microorganisms by use of sanitizing agents, such as chlorine compounds and ozone; 2) to enhance the removal of suspended solids by use of flocculating agents; 3) to remove chlorine compounds; 4) to avoid scaling on reverse osmosis membranes; and 5) to adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute "added substances" as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

5.1.4 ORGANIC SCAVENGERS

Organic scavenging devices use macroreticular, weakly basic anion-exchange resins capable of removing negatively charged organic material and endotoxins from the water. Organic scavenger resins can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity; particulate, chemical, and microbiological fouling of the reactive resin surface; flow rate; regeneration frequency; and shedding of fines from the fragile resins. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

5.1.5 SOFTENERS

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such as calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners can also be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramine disinfectants commonly used in drinking water. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine).

Concerns include microorganism proliferation, channeling, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration.

Control measures involve recirculation of water during periods of low water use; periodic sanitization of the resin and brine system; use of microbial control devices (e.g., UV light and chlorine); locating the unit upstream of the disinfectant removal step (if used only for softening); appropriate regeneration frequency; effluent chemical monitoring (e.g., hardness ions and possibly ammonium); and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then the capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

5.1.6 DEIONIZATION

Deionization (DI) and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. DI systems have charged resins that require periodic regeneration with an acid and base. Typically, cation resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anion resins are regenerated with sodium hydroxide or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, some removal of endotoxin is

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achieved by the anion resin. The system can be designed so that the cation and anion resins are in separate or "twin" beds, or they can be blended together to form a "mixed" bed.

The CEDI system uses a combination of ion-exchange materials such as resins or grafted material, selectively permeable membranes, and an electric charge, providing continuous flow (of product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. The resin acts as a conductor, enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. As the water passes through the resin, it is deionized to become product water. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot achieve the conductivity attribute of Purified Water when starting with the heavier ion load of source water.

Concerns for all forms of DI units include microbial and endotoxin control; chemical additive impact on resins and membranes; and loss, degradation, and fouling of resin. Issues of concern specific to DI units include regeneration frequency and completeness; channeling caused by biofilm agglomeration of resin particles; organic leaching from new resins; complete resin separation for mixed bed regeneration; and bed fluidization air contamination (mixed beds).

Control measures may include continuous recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of bed fluidization air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for DI bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins.

Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors for ensuring proper performance.

5.1.7 REVERSE OSMOSIS

Reverse osmosis (RO) units use semipermeable membranes. The "pores" of RO membranes are intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but they limit the passage of hydrated chemical ions, organic compounds, and microorganisms. RO membranes can achieve chemical, microbial, and endotoxin quality improvement. Many factors, including pH, temperature, source water hardness, permeate and reject flow rate, and differential pressure across the membrane, affect the selectivity and effectiveness of this permeation. The process streams consist of supply water, product water (permeate), and waste water (reject). Depending on the source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve the desired performance and reliability. For most source waters, a single stage of RO filtration is usually not enough to meet Purified Water conductivity specifications. A second pass of this permeate water through another RO stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from source water that has been previously treated with chloramines is removed.

Concerns associated with the design and operation of RO units include membrane materials that are sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; and the passage of dissolved gases, such as carbon dioxide and ammonia. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream; appropriate membrane material selection; membrane design and heat tolerance; periodic sanitization; and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of RO units that can tolerate sanitizing water temperatures and also operate efficiently and continuously at elevated temperatures has added greatly to their microbial control ability and to the avoidance of biofouling. RO units can be used alone or in combination with DI and CEDI units, as well as ultrafiltration, for operational and quality enhancements.

5.1.8 ULTRAFILTRATION

Ultrafiltration is a technology that is often used near the end of a pharmaceutical water purification system for removing endotoxins from a water stream though upstream uses are possible. Ultrafiltration can use semipermeable membranes, but unlike RO, these typically use polysulfone membranes with intersegmental "pores" that have been purposefully enlarged. Membranes with differing molecular weight "cutoffs" can be created to preferentially reject molecules with molecular weights above these ratings.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self-supporting and extremely durable; they can be backwashed, chemically cleaned, and steam sterilized. However, they may require higher operating pressures than do membrane-type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000–20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. As with RO, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filter membrane composition, sanitization, flow design (dead end vs. tangential), cartridge replacement, elevated feed water temperature, and monitoring TOC and differential pressure.

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5.1.9 MICROBIAL-RETENTIVE FILTRATION

Microbial-retentive membrane filters have a larger effective "pore size" than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations.

In water systems, a filter's microbial retention characteristics exhibit different phenomena than in other aseptic filtration applications.

The following factors interact to create the retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes; the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices; and the size and surface properties of the microorganism intended to be retained by the filters. ▲▲ 1S (USP41) In some situations, the appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22-µm rated filters after a ▲▲ 1S (USP41) period of use (days to weeks) seems to support the idea that ▲water-borne microorganisms can penetrate the 0.2- to 0.22-µm rated filters.▲ 1S (USP41) It is not known whether this downstream appearance is caused by ▲exceeding the retentive capabilities of the filters due to high prefiltration bioburden levels of water-borne microorganisms and extended filtration times. These conditions can lead to a "pass-through" phenomenon resulting from tiny cells or less cell "stickiness", or perhaps by a "grow-through" phenomenon in which cells hypothetically replicate their way through the pores to the downstream side.▲ 1S (USP41) Whatever the penetration mechanism, 0.2- to 0.22-µm rated membranes may not be the best choice for some water system uses (see ▲*Sterility Assurance* ⟨1211⟩).▲ (CN 1-May-2018)

Nevertheless, microbial retention success in water systems has been reported with the use of filters rated as 0.2 or 0.1 µm. There is general agreement that, for a given manufacturer, their 0.1-µm rated filters are tighter than their 0.2- to 0.22-µm rated filters. However, comparably rated filters from different manufacturers may not have equivalent performance in water filtration applications because of the different filter materials, different fabrication processes, and nonstandardized microbial retention challenge processes currently used for defining the 0.1-µm filter rating. It should be noted that filters with a 0.1-µm rating may result in a lower flow rate compared to 0.2- to 0.22-µm filters, so whatever filters are chosen for a water system application, the user must verify that they are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between the particles and filter matrix. These electrostatic interactions are so strong, particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product-solution filtrations. These additional adsorptive interactions render filters rated at 0.2–0.22 µm unquestionably suitable for microbial retentive gas filtrations. When microbial retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate. However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use, and periodically thereafter, as well as regular visual inspections, integrity tests, and filter cartridge changes are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters may also be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to "polish" the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling the water upstream of the filters. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see *5.3 Sanitization*), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

5.1.10 ULTRAVIOLET LIGHT

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed in *5.3 Sanitization*, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. At wavelengths around 185 nm (as well as at 254 nm), medium-pressure UV lights have demonstrated utility in the destruction of the chlorine-containing disinfectants used in source water as well as for interim stages of water pretreatment. High intensities of ▲185 nm alone or 254 nm▲ 1S (USP41) in combination with other oxidizing sanitants, such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins.

Areas of concern include inadequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of a UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyperchlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights.

Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb sleeve cleaning and wiping, downstream chlorine detectors (when used for dechlorination), downstream polishing deionizers (when used for TOC reduction), and regular (approximately yearly) bulb replacement. UV lamps generate heat during operation, which can cause failure of the lamps or increase the temperature of the water. Precautions should be in place to ensure that water flow is present to control excessive temperature increase.

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5.1.11 DISTILLATION

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available, including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Source water controls must provide for the removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces, as well as the removal of those impurities that could volatize and condense along with the water vapor. In spite of general perceptions, even the best distillation process does not ensure absolute removal of contaminating ions, organics, and endotoxins. Most stills are recognized as being able to accomplish at least a 3–4 log reduction in these impurity concentrations. They are highly effective in sterilizing the feed water.

Areas of concern include carryover of volatile organic impurities such as trihalomethanes (see *2. Source Water Considerations*) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blow down, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve the following: preliminary steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feed water droplet entrainment; visual or automated high-water-level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feed water and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still start-up or still malfunction from getting into the finished water distribution system; and periodic testing for pinhole leaks to routinely ensure that condensate is not compromised by nonvolatized source water contaminants.

5.1.12 STORAGE TANKS

Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the purification system while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity.

Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from unalarmed rupture disk failures caused by condensate-occluded vent filters.

Control considerations may include using closed tanks with smooth interiors, the ability to spray the tank headspace using spray balls on recirculating loop returns, and the use of heated, jacketed/insulated tanks. This minimizes corrosion and biofilm development and aids in thermal or chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be accomplished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank.

5.1.13 DISTRIBUTION SYSTEMS

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of no recirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be flushed periodically and monitored more closely. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot-water sanitized systems) as well as thorough chemical sanitant distribution. Turbulent flow also appears to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant components, such as pumps or filters, are used, they should be configured and used to avoid microbial contamination of the system.

Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In distribution systems, dead legs and low-flow conditions should be avoided, and valved tie-in points should have length-to-diameter ratios of six or less. In systems that operate at self-sanitizing temperatures, precautions should be taken to avoid cool points where biofilm development could occur. If drainage of components or distribution lines is intended as a microbial control strategy, they should also be configured to be dried completely using dry compressed gas because drained but still moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification system.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use to deliver water must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

5.1.14 NOVEL/EMERGING TECHNOLOGIES

New water treatment technologies are being developed continuously. Before these technologies are utilized in pharmaceutical water systems, they should be evaluated for acceptable use in a GMP environment. Other considerations should include the treatment process, reliability and robustness, use of added substances, materials of construction, and ability to validate. Consideration should be given to recognize the areas of concern during the evaluation and to identify control measures for the technology. This should include impact on chemical and microbial attributes.

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5.2 Installation, Materials of Construction, and Component Selection

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation should promote gravity drainage. Pipe supports should provide appropriate slopes for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components—including units of operation, tanks, and distribution piping—require careful attention to preclude potential operational and microbial problems.

Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation of metal surfaces after installation are important for removing contamination and corrosion products and to re-establish the passive corrosion-resistant surface.

Plastic materials can be fused (welded) in some cases, and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and organic extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Use of plastic materials may contribute to TOC levels.

Control measures include good alignment, properly sized gaskets, appropriate spacing, uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, or passivation. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. If chemicals or additives will be used to clean, passivate, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without erosion of the corrosion-resistant film (such as the passive chromium oxide surface of stainless steel) or reduction in wall thickness for plastics. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement the system design and provide satisfactory corrosion and microbial activity resistance. The finish should also be a material that can be chemically sanitized. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as the manufacturer's metallurgical reports for stainless steel and reports of composition, ratings, and material-handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference. Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination intrusion. Heat exchangers should be constructed to prevent leakage of heat transfer medium into the pharmaceutical water and, for heat exchanger designs where prevention may fail, there should be a means to detect leakage. Pumps should be of sanitary design with seals that prevent contamination of the water. Valves should have smooth internal surfaces with the seat and closing device exposed to the flushing action of water, such as occurs in diaphragm valves. Valves with pocket areas or closing devices (e.g., ball, plug, gate, globe) that move into and out of the flow area should be avoided.

5.3 Sanitization

Microbial control in water systems is achieved primarily through sanitization practices. Systems can be sanitized using either thermal or (photo-)chemical means.

5.3.1 THERMAL SANITIZATION

Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of 65°–80° are most commonly used for thermal sanitization. Continuously recirculating water of at least 65° at the coldest location in the distribution system has also been used effectively in stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Frequent use of thermal sanitization at appropriate temperatures should eliminate the need for other sanitization methods.

The use of thermal methods at temperatures above 80° is contraindicated because it does not add to microbial control of the system or reduction of biofilm. Some methods (e.g., steam sanitizing, hot water circulation at temperatures ≥100°) can be less effective or even destructive because of the need to eliminate condensate or manipulate system components, stress materials of construction, deform filters, and its adverse impact on instrumentation.

Although thermal methods control biofilm development by either continuously inhibiting its growth or, in intermittent applications, by killing the microorganisms within developing biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In cases of infrequent thermal sanitizations that allow biofilm development between treatments, a combination of routine thermal treatment and periodic supplementation with chemical sanitization may be more effective. The more frequent the thermal sanitization, the more likely it is that biofilm re-development can be eliminated.

5.3.2 CHEMICAL SANITIZATION

Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically use oxidizing agents such as ozone, hydrogen peroxide, peracetic acid, or combinations thereof. Halogenated compounds can be effective sanitizers but are less aggressive oxidizing agents and may be difficult to flush from the system. Chemical agents may not penetrate the full biofilm matrix or extend into all biofilm locations (such as crevices at gasketed fittings) and may

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leave biofilms incompletely inactivated. Compounds such as ozone, hydrogen peroxide, and peracetic acid oxidize bacteria and biofilms with reactive peroxides and by forming very reactive free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations, require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and/or oxygen, and peracetic acid degrades to oxygen and acetic acid. The ease of degradation of ozone to oxygen using 254-nm UV lights in circulating loops allows it to be used effectively on a continuously sanitizing basis in holding tanks and on an intermittent basis (e.g., daily or weekly) in the distribution loops. The highly reactive nature of ozone requires the use of system materials and components that are even more oxidation resistant than those typically used with the other oxidizing agents.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by using aggressive oxidizing chemicals. The less developed and therefore thinner the biofilm, the more effective the biofilm inactivation. Therefore, optimal microbial control is achieved by using oxidizing chemicals at a frequency that does not permit significant biofilm development between treatments.

Validation of chemical sanitization requires demonstration of adequate chemical concentrations throughout the system, exposure to all wetted surfaces including the body of use point valves, and complete removal of the sanitant from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the sanitant or its objectionable degradants is an essential part of the validation program.

5.3.3 UV SANITIZATION

In-line UV light at a wavelength of 254 nm can also be used to continuously "sanitize" only the water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbially retentive filter, UV light is most effective and can prolong the interval between needed system re-sanitizations.

5.3.4 SANITIZATION PROCEDURES

Sanitization steps require validation to demonstrate the ability to reduce and hold microbial contamination at acceptable levels. Validation of thermal methods should include a heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves; sampling ports; instrument side branches; and fittings, couplings, and adapters, relying on water convection and thermal conduction through system materials for heat transfer to wetted surfaces.

The routine frequency of sanitization should be supported by the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for the need for extraordinary maintenance. The routine frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not regularly exceed Alert and Action Levels (see *9.4 Defining Alert and Action Levels and Specifications*).

5.4 Operation, Maintenance, and Control

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include 1) procedures for operating the system, 2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, 3) schedule for periodic sanitization, 4) preventive maintenance of components, and 5) control of changes to the mechanical system and to operating conditions.

5.4.1 OPERATING PROCEDURES

Operating procedures for the water system and for performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented, and should detail the function of each job, assign who is responsible for performing the work, describe how the job is to be done, and identify acceptable operating parameters. The effectiveness of these procedures should be assessed during water system validation.

5.4.2 PROCESS MONITORING PROGRAM

A process-monitoring program should establish the critical quality attributes and operating parameters that are documented and monitored. The program may include a combination of in-line sensors and/or automated instruments (e.g., for temperature, TOC conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (e.g., flow rates or pressure drop across a carbon bed, filter, or RO unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity of initiating corrective action should be included.

5.4.3 ROUTINE MICROBIAL CONTROL

Sanitization may be integral to operation and maintenance, and necessary on a routine basis, depending on system design and the selected units of operation, to maintain the system in a state of microbial control. Technologies for sanitization are described above in more detail in *5.3 Sanitization*.

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5.4.4 PREVENTIVE MAINTENANCE

A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

5.4.5 CHANGE CONTROL

The mechanical configuration, operating conditions, and maintenance activities of the water system must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to requalify the system after changes are made should be determined. After a decision is made to modify a water system, the affected drawings, manuals, and procedures should be revised. Portions or operations of the water system that are affected by the modification should be tested to demonstrate a continued state of control. The extent and duration of testing should be related to the risk impact of the change to the system.

Change to read:

6. SAMPLING

The testing of water samples from a water system is critical to the ongoing control of the system and assessment of the quality of the water being used. If improperly collected, a sample could yield a test result that is unrepresentative of the sample's purpose. This could lead to inaction when remediation is needed or to unnecessary remediation when none is necessary. It could also lead to misinterpretations of product impact. Therefore, properly collecting water samples, understanding their purpose, and establishing appropriate water system sampling plans are essential to water quality control and system control.

6.1 Purposes and Procedures

To assess a particular water ▲▲ 1S (USP41) attribute, a sample of the water usually must be removed from a water system for specific ▲▲ 1S (USP41) attribute testing. ▲The sample needs to be obtained from specific locations that are representative for the purpose being monitored.▲ 1S (USP41) This sample may be analyzed by in-line/on-line instruments or it may be completely removed from the system as a "grab sample" in a container for off-line testing. In-line/on-line testing avoids the exogenous contamination potential of grab samples that could lead to artifactually variable data trends and incorrect decisions on system performance, maintenance, and utilized ▲water,▲ 1S (USP41) as well as initiating fruitless causative investigations. ▲Grab samples may be appropriate where the water in the system is not homogeneous for certain attributes.▲ 1S (USP41)

The data from water testing are generally used for one of two purposes: for process control (PC) of the water purification and distribution system or for ▲release or▲ 1S (USP41) QC of the water being drawn from the system for some application or use. In many cases, depending on the sampling location and sampling process, the resulting data can be used for both PC and QC purposes.

6.1.1 PC SAMPLING

Because PC sampling is intended to reflect the quality of the water behind the valve and within the distribution system, coming from the purification system, or between its purification steps, efforts should be made to avoid contaminating the water as it ▲is▲ 1S (USP41) drawn from the system so that its test results accurately reflect the water quality within the system at that location. This may require the use of strategically located sampling ports, in addition to points of use.

If microbial testing is needed for PC purposes, the sampling valve should have a properly installed, sanitary design that uses vigorous pre-sampling flushing. This flushing shears off fragile biofilm structures growing on surfaces within the valve and water path before the sample is collected. This avoids biasing the microbial count of perhaps pristine water in the system behind that valve. A fully open valve flush (at >8 ft/s velocity within the valve and connector) for at least 30 s typically provides sufficient shear forces to adequately remove any fragile biofilm structures. Additional control measures for preventing sample contamination could also include stringent pre- and post-sampling outlet sanitation, the use of sterile hoses and gaskets or other connectors to direct the water flow, and other measures.

The data from PC sampling indicate how well the system is maintaining the water quality at that sampling location. These data are subsequently used to signal when some extraordinary intervention might be needed, in addition to normal maintenance and system sanitization operations, to restore the system to the expected level of purity.

PC sampling can only be used to indicate the quality of the water being delivered to the points of use (for QC purposes) if it has been shown to be representative of that point-of-use quality. This may be possible with chemical attributes that are typically not affected by the fluid path of the water delivery process, but is generally not possible with microbial attributes, which can be greatly affected by localized biofilms along that fluid path. If this fluid path is not utilized for PC sampling, then the resulting data typically cannot be used for QC purposes.

6.1.2 QC SAMPLING

QC sampling is intended to reflect the quality of water that is being used. These samples should be collected at the true point of use; that is, where the water is delivered for use, not where it leaves the water system. QC sampling must utilize that same delivery path and components utilized for a water transfer during actual water use. This includes the same valves, hoses, heat exchangers, flow totalizers, hard-piped connections, and other components utilized during water use.

In addition to the water transfer components, QC sampling must also use the same water transfer process employed during water use, including the same pre-use outlet and delivery path flushing procedure and the same outlet, fitting, and hose

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sanitization practices employed during actual water use. The water delivery process and components used for QC sampling must be identical to manufacturing practices at every system outlet for the QC sample to mimic the quality of water being used by accumulating the same chemical and microbial contaminant levels it would during actual use from that outlet location.

Where permanent connections from the water system to equipment are present, accommodation should be made in the design to collect samples from locations as close to the equipment as possible. For example, samples can be collected from special sample ports or other valves near the equipment connection that allow the collected water sample to accurately reflect the water quality that is used. Where the water transfer conduit is designed and/or definitively treated to eliminate all contaminating influences prior to water transfer through that conduit, PC sampling locations within the distribution system can reflect the quality of the water that is actually used for QC purposes at those permanent connections. However, the success of the design and treatments intended to eliminate these contaminating influences must be verified. This is typically done during water system validation.

Where routine water use practices involve contamination-prone activities, such as no pre-use flushing or poor hose storage/sanitization/replacement practices, these water use practices should be improved to reduce the potential for delivering contaminated water from the water system and for unacceptable QC sample testing results that reflect that same contamination.

6.2 Attributes and Sampling Locations

The tests being performed on the samples are relevant to the sampling location and purpose of the sample. In-process monitoring of nonmonograph attributes may be indicated for specific unit operations. For instance, before and after a softener, it may be important to determine water hardness to verify softener efficacy. Before and after an activated carbon bed/filter, it may be important to verify chlorine or TOC removal and/or reduction or test for an increase in microbial count. Before a distillation unit, it may be important to quantitate the incoming bacterial endotoxin level to ensure that the still is not being over-challenged beyond its typical 3–4 log purification capability. However, once the water is in the distribution system, the compendial attributes of importance typically include at least conductivity, TOC, and microbial count. In Water for Injection systems and other systems or system locations where bacterial endotoxin control is important, endotoxin is also assayed. Other tests may be necessary depending on the intended uses of the water.

6.2.1 CHEMICAL ATTRIBUTES

Dissolved chemical contaminants detected by conductivity or TOC testing tend to be uniformly distributed in the water throughout the water system. However, there are exceptions where localized chemical contamination sources can occur, such as from a coolant-leaking heat exchanger in a sub-loop, or at a point of use, or within a dead leg. These chemical contaminants may only be seen at the associated outlets and not systemically. However, in the absence of localized contamination influences, chemical attributes are candidates for on-line testing at fixed strategic locations within the distribution system, such as near a circulating loop return, and are generally reflective of the same chemical quality at all locations and points of use within the distribution system. Nevertheless, the suitability of the on-line locations of these instruments for QC release purposes must be verified as being representative of the use-point water quality. This is usually done during water system validation.

6.2.2 MICROBIAL ATTRIBUTES

The same uniformity scenario cannot be assumed for microbial attributes. Planktonic organisms in a water sample could have originated from biofilms in the purification or distribution systems releasing more or less uniform levels of planktonic organisms into the circulating water, as detectable in samples from all outlets. However, a local biofilm developing within a water delivery conduit (such as a use-point outlet valve and transfer hose) in an otherwise pristine biofilm-free water system could release planktonic organisms detectable only in water delivered through that conduit. Therefore, QC release samples for assessing the quality of water that is delivered by the system during water use must be collected after the water has traversed the same fluid conduit (including the same preparatory activities such as outlet sanitization and pre-flushing) from the water distribution system to the specific locations where the water is used.

On-line microbial water sampling/testing has value in pharmaceutical water systems only for PC purposes unless the water is taken from the point of use in the same manner as routine water usage, in which case the data can also have a QC release purpose. Microbial counts detected from strategic sampling ports continue to have PC and investigational value, but generally cannot be substituted for QC release testing except in certain scenarios, as described in *6.1.2 QC Sampling*.

6.3 Validation Sampling Plans

The initial sampling plan for a pharmaceutical water system is usually developed for a validation program (see *4. Validation and Qualification of Water Purification, Storage, and Distribution Systems*). This strategy is for characterization of the system's ability to purify, distribute, and deliver pharmaceutical water. Typically, the initial validation sampling is for a short duration (e.g., at least 2–4 weeks) at a high sampling frequency to generate a significant body of data that will allow detection of short-term or localized chemical or microbial quality deviations from all outlets. These data provide an initial assessment of system performance to guide decisions about using the water for operational purposes.

The initial validation sampling plan is re-evaluated when the pharmaceutical water is placed into operation, typically to reduce the amount of data being generated while not compromising the ability to identify anomalous operations/events, especially during the early life cycle of the water system. In the absence of such quality deviations during the initial sampling period, the sampling frequency can be lessened for a period of time (e.g., at least 2–4 additional weeks) to ensure that somewhat longer-term adverse quality trends are not apparent. During this second period of time, the water may be considered for at-risk routine use, pending the acceptable completion of the second validation sampling period. After successful completion, monitoring can eventually be lessened again to what will become the routine sampling plan.

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Periodic review of the water system operation and monitoring needs to be performed to assess seasonal source water variability, effectiveness of sanitization, and routine maintenance events. Periodic review should be performed during the complete life cycle of the water system, typically annually, for evidence of longer-term data trends and quality deviations.

The routine sampling plan should be re-evaluated periodically based on the available data to determine the appropriate frequency and sample locations. This review offers an opportunity to improve data evaluation and reduce workloads based on what that data indicate relative to process and quality control. The routine sampling plan should have a rationale for the frequency and locations that are selected to justify how the resulting data will be used to characterize the overall operation of the system and the release of the water for use.

6.4 Routine Sampling Plans

6.4.1 SOURCE WATER SAMPLING

As mentioned in earlier sections, the source water for pharmaceutical water systems must comply with the standards for one of the Drinking Waters listed in the associated compendial water monograph or in *General Notices*. When a municipality or other water authority is providing this Drinking Water, they are required to comply with the local Drinking Water Regulations for the water supplied to a drinking or potable water distribution piping grid for that region. The quality of that water by the time it reaches the pharmaceutical user is dependent on a number of factors including distance from the input source, duration of travel within the piping, and condition of the piping in that potable water distribution grid, any of which could have adversely affected some of its initial chemical and/or microbial attributes. Based on a risk assessment, it may be prudent to verify full compliance with regulations using water collected from sample ports prior to the pretreatment system, or other equivalent Drinking Water outlets within the facility. If the water complies, then continued assurance of compliance could be verified using Drinking Water Regulation test results provided by the water authority or by periodic retesting of selected or all the Drinking Water attributes by the user or by both the user and the water authority. If private sourced water is utilized, it is the user's responsibility to demonstrate full Drinking Water regulation compliance, using water samples from such sampling ports on a periodic basis as determined by a risk analysis.

These pre-pretreatment sampling ports could, at the user's discretion, be used to periodically monitor other source water attributes that could affect specific pretreatment or purification unit operations. Depending on the user's source water quality consistency and a risk assessment of its potential impact on the purification process, the periodically monitored attributes could include microbial count, absence of coliforms, bacterial endotoxin levels, conductivity, TOC, pH, hardness, chlorine, silica, turbidity or silt density index, and others. These data could be useful in investigations and for operational adjustments to critical unit operation parameters and maintenance procedures, or for feedback to the potable water provider if unusual trends are observed.

6.4.2 PRETREATMENT AND PURIFICATION SYSTEM SAMPLING

The location and frequency of sampling from ports within the pretreatment and purification systems may be selected based on a risk analysis of unit operation purpose. The purpose of this sampling is primarily for PC, for example, to ensure maintenance of acceptable unit operation performance, to assess maintenance procedure efficacy, and to investigate the need for remedial action. Quality deviations in the early portions of the purification process can affect unit operation efficiency but usually do not impact the finished water quality or acceptable use.

6.4.3 PURIFIED WATER DISTRIBUTION SYSTEM SAMPLING

Purified Water distribution system sampling is intended to provide continuing assurance of ongoing PC and compliance with the user's finished water chemical and microbiological requirements. Generally, the locations for that sampling and the frequency of testing the specific attributes are a matter of process and quality control consistency, as well as risk tolerance in the event of a deviation.

Depending on the water system design, the chemical attributes of a water system tend to be relatively constant and more uniformly distributed than the microbiological attributes. Therefore, less frequent sampling at only selected locations could be justified for chemical testing based on familiarity with system design and the existence of historically consistent operational data. However, with some purification system designs, the chemical quality could change dramatically in a short period of time (such as from the exhaustion of deionization beds), so frequent or even continuous in-line/on-line monitoring of the chemical attributes would be advisable to be able to recognize and correct the cause of the problem before non-compliant water is produced and used.

For microbial testing, all use points and critical sample ports in a distribution system are typically sampled routinely, including those that are infrequently used by manufacturing. There is no prescribed sampling frequency for Purified Water system outlets, so typical outlet sampling frequencies vary from daily to monthly, with sampling occurring somewhere in the system at least at weekly intervals.

A risk analysis is suggested for determining the sampling plan for a Purified Water system. Factors in this analysis could include (but are not limited to) the test result history for the entire water system as well as specific outlets, the criticality of specific outlets to manufacturing, the usefulness of selected sample ports as indicators of ongoing system control, and the scope of impact on products and activities should an unfavorable test result occur. For the scope of impact, the less frequent the sampling, the more products and processes will be impacted by an unfavorable test result.

6.4.4 WATER FOR INJECTION DISTRIBUTION SYSTEM SAMPLING

The sampling plans for Water for Injection distribution systems (as well as any water system where some level of bacterial endotoxin control is needed) utilize the same general sampling approaches as do Purified Water systems. However, the

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regulatory expectations for Water for Injection distribution system sampling plans are more prescriptive because microbial control must be much more stringent as it is related to the bacterial endotoxin attribute. In general, water sampling for microbial and bacterial endotoxin testing is expected to occur daily somewhere in the system, with each outlet being sampled periodically, based on a risk assessment, to characterize the quality of the water.

6.5 Non-Routine Sampling

Non-routine sampling can also be performed on the water system for episodic events or reasons for which the routine sampling plans are insufficient to capture the needed information. Examples include change control purposes such as evaluating potential changes to sampling, testing, maintenance procedures, or system design; data or event excursion investigation purposes; or simply for long-term informational purposes and establishing baselines for future investigational value. The purpose of the non-routine sampling dictates the sampling procedures to be used, the attributes to be tested, and the location and repeating occurrence (if any) of that testing. It should also be noted that such non-routine sampling may be done from sampling ports that may or may not be routinely tested. Sampling ports can be positioned in a water system purely for investigational, non-routine sampling, and as such, they do not need to be part of a routine sampling plan.

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7. CHEMICAL EVALUATIONS

7.1 Chemical Tests for Bulk Waters

The chemical attributes of *Purified Water* and *Water for Injection* that were in effect prior to *USP 23* were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. Although these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

In 1996, USP moved away from these chemical attribute tests, switching to contemporary analytical technologies for the bulk waters Purified Water and Water for Injection. The intent was to upgrade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for *Oxidizable Substances* that primarily targeted organic contaminants. A multi-staged conductivity test that detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for *Heavy Metals*, all of the inorganic chemical tests (i.e., *Ammonia*, *Calcium*, *Carbon Dioxide*, *Chloride*, *Sulfate*).

Replacing the heavy metals attribute was considered unnecessary because 1) the source water specifications (found in the U.S. EPA's NPDWR) for individual heavy metals were tighter than the approximate limit of detection of the *Heavy Metals* test for *USP XXII* Water for Injection and Purified Water (approximately 0.1 ppm), 2) contemporary water system construction materials do not leach heavy metal contaminants, and 3) test results for this attribute have uniformly been negative; there has not been a confirmed occurrence of a singular test failure (failure of only the *Heavy Metals* test with all other attributes passing) since the current heavy metal drinking water standards have been in place.

Total Solids and *pH* were the only tests not covered by conductivity testing. The test for *Total Solids* was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in Purified Water and Water for Injection is easily removed by most water pretreatment steps, and even if present in the water, it constitutes no medical or functional hazard except in extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user's responsibility to ensure fitness for use. If silica is a significant component in the source water, and the purification unit operations could fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants detectable by conductivity), then either silica-specific testing or a total-solids type testing should be utilized to monitor for and control this rare problem.

The *pH* attribute was eventually recognized to be redundant to the conductivity test (which included *pH* as an aspect of the test and specification); therefore, *pH* was discontinued as a separate attribute test.

The rationale used by USP to establish its Purified Water and Water for Injection conductivity specifications took into consideration the conductivity contributed by the two least-conductive former attributes of *Chloride* and *Ammonia*, thereby precluding their failure had those wet chemistry tests been performed. In essence, the *Stage 3* conductivity specifications (see *Water Conductivity* ⟨645⟩, *Bulk Water, Procedure, Stage 3*) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water (H^+ and OH^-), dissolved atmospheric carbon dioxide (as HCO_3^-), and an electro-balancing quantity of either sodium (Na^+) or chlorine (Cl^-), depending on the pH-induced ionic imbalance (see *Table 1*). The *Stage 2* conductivity specification is the lowest value in this table, 2.1 µS/cm. The *Stage 1* specifications, designed primarily for on-line measurements, were derived by essentially summing the lowest values in ▲individual (H^+, OH^-, HCO_3^-) and group (Cl^-, Na^+, NH_4^+) of▲ 1S (USP41) contributing ion columns for each of a series of tables similar to *Table 1*, created for each 5° increment between 0° and 100°. For example purposes, the italicized values in *Table 1*, the conductivity data table for 25°, were summed to yield a conservative value of 1.3 µS/cm, the *Stage 1* specification for a nontemperature-compensated, nonatmosphere-equilibrated water sample that actually had a measured temperature of 25°–29°. Each 5° increment in the table was similarly treated to yield the individual values listed in the table of *Stage 1* specifications (see *Water Conductivity* ⟨645⟩, *Bulk Water*).

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Table 1. Contributing Ion Conductivities of the Chloride-Ammonia Model as a Function of pH (in atmosphere-equilibrated water at 25°)

	Conductivity (µS/cm)							
pH	H^+	OH^-	HCO_3^-	Cl^-	Na^+	NH_4^+	Combined Conduct-ivities	Stage 3 Limit
5.0	3.49	*0*	*0.02*	*1.01*	*0.19*	*0*	4.71	4.7
5.1	2.77	0	0.02	1.01	0.29	0	4.09	4.1
5.2	2.20	0	0.03	1.01	0.38	0	3.62	3.6
5.3	1.75	0	0.04	1.01	0.46	0	3.26	3.3
5.4	1.39	0	0.05	1.01	0.52	0	2.97	3.0
5.5	1.10	0	0.06	1.01	0.58	0	2.75	2.8
5.6	0.88	0	0.08	1.01	0.63	0	2.60	2.6
5.7	0.70	0	0.10	1.01	0.68	0	2.49	2.5
5.8	0.55	0	0.12	1.01	0.73	0	2.41	2.4
5.9	0.44	0	0.16	1.01	0.78	0	2.39	2.4
6.0	0.35	0	0.20	1.01	0.84	0	2.40	2.4
6.1	0.28	0	0.25	1.01	0.90	0	2.44	2.4
6.2	0.22	0	0.31	1.01	0.99	0	2.53	2.5
6.3	0.18	0	0.39	0.63	0	1.22	2.42	2.4
6.4	0.14	0.01	0.49	0.45	0	1.22	2.31	2.3
6.5	0.11	0.01	0.62	0.22	0	1.22	2.18	2.2
6.6	0.09	0.01	0.78	0	0.04	1.22	2.14	2.1
6.7	0.07	0.01	0.99	0	0.27	1.22	2.56	2.6
6.8	0.06	0.01	1.24	0	0.56	1.22	3.09	3.1
6.9	0.04	0.02	1.56	0	0.93	1.22	3.77	3.8
7.0	*0.03*	0.02	1.97	0	1.39	1.22	4.63	4.6

As stated above, this rather radical change to utilizing a conductivity attribute as well as the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed industry to realize substantial savings. The TOC and conductivity tests can also be performed off-line in the laboratories using collected samples, although sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data, where previously only a single data point was available. As stated in *6. Sampling*, continuous in-process data are excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but this is too much data for QC purposes. Therefore, for example, one can use a justifiable portion of the data (at a designated daily time or at the time of batch manufacturing) or the highest value in a given period as a worst case representation of the overall water quality for that period. Data averaging is generally discouraged because of its ability to obscure short-lived extreme quality events.

7.2 Chemical Tests for Sterile Waters

Packaged/sterile waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the major source of chemicals (inorganics and organics) that leach over time into the packaged water and can easily be detected by the conductivity and TOC tests. The irony of organic leaching from plastic packaging is that before the advent of bulk water TOC testing, when the *Oxidizable Substances* test was the only "organic purity" test for both bulk and packaged/sterile water monographs in *USP*, the insensitivity of that test to many of the organic leachables from plastic and elastomeric packaging materials was largely unrecognized, allowing organic levels in packaged/sterile water to be quite high (possibly many times the TOC specification for bulk water).

Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity but poorly detected by the former wet chemistry attribute tests. Most of these leachables are considered harmless based on current perceptions and standards at the rather significant concentrations present. Nevertheless, they effectively degrade the quality of the high-purity waters placed into these packaging systems. Some packaging materials contain more leachables than others and may not be as suitable for holding water and maintaining its purity.

The attributes of conductivity and TOC tend to reveal more about the packaging leachables than they do about the water's original purity. These currently "allowed" leachables could render the sterile packaged versions of originally equivalent bulk water essentially unsuitable for many uses where the bulk waters are perfectly adequate.

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Therefore, to better control the ionic packaging leachables, ⟨645⟩ is divided into two sections. The first, *Water Conductivity* ⟨645⟩, *Bulk Water*, applies to *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and *Pure Steam*, and includes the three-stage conductivity testing instructions and specifications. The second, *Water Conductivity* ⟨645⟩, *Sterile Water*, applies to *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*. The *Sterile Water* section includes conductivity specifications similar to the *Water Conductivity* ⟨645⟩, *Bulk Water, Procedure, Stage 2* testing approach because it is intended as a laboratory test, and these sterile waters were made from bulk water that already complied with the three-stage conductivity test. In essence, packaging leachables are the primary target analytes of the conductivity specifications in *Water Conductivity* ⟨645⟩, *Sterile Water*. The effect on potential leachables from different container sizes is the rationale for having two different specifications, one for small packages containing nominal volumes of 10 mL or less and another for larger packages. These conductivity specifications are harmonized with the *European Pharmacopoeia* conductivity specifications for Sterile Water for Injection. All monographed waters, except *Bacteriostatic Water for Injection*, have a conductivity specification that directs the user to either the *Bulk Water* or the *Sterile Water* section. For the sterile packaged water monographs, this water conductivity specification replaces the redundant wet chemistry limit tests intended for inorganic contaminants that had previously been specified in these monographs.

Controlling the organic purity of these sterile packaged waters, particularly those in plastic packaging, is more challenging. Although the TOC test can better detect these impurities and therefore can be better used to monitor and control these impurities than the current *Oxidizable Substances* test, the latter has a history of use for many decades and has the flexibility to test a variety of packaging types and volumes that are applicable to these sterile packaged waters. Nevertheless, TOC testing of these currently allowed sterile, plastic-packaged waters reveals substantial levels of plastic-derived organic leachables that render the water perhaps orders of magnitude less organically pure than is typically achieved with bulk waters. Therefore, usage of these packaged waters for analytical, manufacturing, and cleaning applications should only be exercised after the purity of the water for the application has been confirmed as suitable.

▲7.3 Storage and Hold Times for Chemical Tests

Due to the homogeneous nature of chemical impurities in water, unlike the challenges of microbial impurities, the storage requirements and impact of holding times are very practically determined. In general, the chemical purity of high-purity water samples can only degrade over time, possibly generating a failed result of the sample that would have passed if it were tested immediately or on-line. The general fact is that the longer samples are stored, the greater the potential to be adversely impacted by containers or conditions.

For off-line chemical tests of waters, there are no compendial requirements for storage time and conditions. However, the general recommendation is to perform testing as soon as practical to avoid false adverse results. Where possible, store cool and measure as quickly as practical. This reduces the chances that a water sample gets contaminated over time, and this would reduce unwarranted and unnecessary investigations of false positives.

7.3.1 CONTAINERS

When sampling water for off-line analysis, the selection and cleanliness of the container play a significant part in obtaining accurate data. For samples to be tested for chemical impurities according to ⟨645⟩ and *Total Organic Carbon* ⟨643⟩, the proper container should be one that does not contaminate the sample during the storage/hold time. For example, the use and preparation of glass containers could be very acceptable for storing samples for TOC testing, but some glass containers do leach ions over time (hours and days), and they can adversely impact a conductivity test by creating a false positive result—if the storage time is too long. Likewise, there are some polymer materials that can adversely impact the TOC chemical impurity in water. However, many polymer materials are very inert.

In any case, cleanliness of the container is crucial because trace quantities of soaps and fingerprints will adversely impact the chemical purity of the water. Properly cleaned containers are acceptable because chemical impurities are easily rinsed away. Extensive chemical cleaning methods such as acid or caustic rinsing should never be needed. If they are needed, consider replacing the containers.

7.3.2 STORAGE TIME AND CONDITIONS

There are no specific recommendations for storage of samples for water analyses. If there is some trace interaction of the container and water, then generally colder and shorter storage times are better than warmer and longer storage times. Chemical dissolution and reactivity are usually enhanced by increased temperature. Furthermore, time is always an element because the water sample can only get worse in a container, and it never gets better with time.

7.4 Elemental Impurities in Pharmaceutical Waters

Elemental impurities (EI) have the most restrictive limits for Water for Injection used in manufacturing parenterals, in particular large-volume injections (see *Injections and Implanted Drug Products* ⟨1⟩ for a definition of large-volume injections) because of the large dose. The most restrictive permissible daily exposure (PDE) of EI resides with lead, mercury, cadmium, and arsenic. Other EI listed in ⟨232⟩ permit a substantially higher PDE, and are therefore less restrictive.

Water that meets U.S. EPA National Primary Drinking Water Regulations or WHO Drinking Water Guidelines that has been purified by conventional technologies used to produce Water for Injection can comply with ⟨232⟩ for parenterals.

Table 2 shows that source water that meets US EPA NPDWR or WHO Drinking Water Guidelines has maximum contaminant levels (concentration) for lead, mercury, cadmium, and arsenic that are NMT 10 times (1-log) higher than the EI limits for parenterals, based on a daily dose of 2000 mL. For a smaller volume injection, the allowed parenteral daily dose of EI is correspondingly higher. The purification technologies needed to produce Water for Injection that reduce the impurities by a

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factor of 100 to 1000 will assure compliance with ⟨232⟩, provided there are no elemental impurities added during processing, packaging, delivery, or storage.

Table 2. Elemental Impurity Limits for Drug Products and their Water Components per ⟨232⟩

Element	Parenteral PDE (µg/day)	Parenteral Daily Dose (µg/mL[a])	U.S. EPA National Primary Drinking Water Regulations (µg/mL[b])	WHO Drinking Water Guidelines (µg/mL[b])	Result of 2-Log Reduction of EI Concentration for WFI (µg/mL[c])
Cadmium	2	0.001	0.005	0.003	0.00005
Lead	5	0.0025	0.015	0.01	0.00015
Inorganic arsenic	15	0.0075	0.01	0.01	0.0001
Inorganic mercury	3	0.0015	0.002	0.006	0.00006

[a] Concentration based on a daily dose of 2000 mL, and all drug product elemental impurities coming from the water component.
[b] Drinking Water Regulations state these Maximum Contaminant Levels (MCLs) as mg/L, which equals µg/mL or ppm.
[c] Determined from the greater of the US EPA Regulations column and WHO Guidelines column for each element, then divided by 100 (2–log).

Chemical purification technologies for Purified Water are similarly efficient in removing EI as those for Water for Injection production. Because all sterile waters are prepared from Purified Water or Water for Injection, the assurance of compliance to ⟨232⟩ extends to sterile waters, provided there are no elemental impurities added during processing, packaging, delivery, or storage.

Further discussion can be found in *Pharmacopeial Forum* [see Bevilacqua A, Soli TC, USP Chemical Analysis Expert Committee. Elemental impurities in pharmaceutical waters. *Pharm Forum.* 2013;39(1)]. ▲1S (USP41)

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8. MICROBIAL EVALUATIONS

This section of the chapter presents a discussion about the types and sources of microorganisms and whether certain microbes are prone to colonize pharmaceutical water systems. This section also addresses microbiological examination of water samples, including a discussion on recovery methods.

8.1 Microorganism Types

Microorganisms are ubiquitous and their natural habitats are extremely diverse. Based on comparative ribosomal RNA sequencing, the phylogenetic tree of life consists of three domains: Bacteria and Archaea (both prokaryotes), and Eukarya (eukaryotes). Most microorganisms that contaminate pharmaceutical products are prokaryotic bacteria and eukaryotic fungi (yeasts and molds). These microbes are typical isolates from pharmaceutical environments, including the associated personnel, and a few are frank or opportunistic pathogens. Contamination with viruses is a concern in bioprocessing that uses animal cells.

8.1.1 ARCHAEANS

Microbes from the domain Archaea are phylogenetically related to prokaryotes but are distinct from bacteria. Many are extremophiles, with some species capable of growing at very high temperatures (hyperthermophiles) or in other extreme environments beyond the tolerance of any other life form. In general, most extremophiles are anaerobic or microaerophilic chemolithoautotrophs. Because of their unique habitats, metabolism, and nutritional requirements, Archaeans are not known to be frank or opportunistic pathogens, and they are not capable of colonizing a pharmaceutical water system.

8.1.2 BACTERIA

Bacteria are of immense importance because of their rapid growth, mutation rates, and ability to exist under diverse and adverse conditions; ▲some of them are human pathogens.▲1S (USP41) Some are very small and can pass through 0.2-µm rated filters. Others form spores, which are not part of their reproductive cycle. Bacterial spore formation is a complex developmental process that allows the organisms to produce a dormant and highly resistant cell in times of extreme stress. Bacterial endospores can survive high temperatures, strong UV irradiation, desiccation, chemical damage, and enzymatic destruction, which would normally kill vegetative bacteria.

Using a traditional cellular staining technique based on cell wall compositional differences, bacteria are categorized into Gram positive and Gram negative, although many sub-groups exist within each category based on genomic similarities and differences.

8.1.2.1 Gram-positive bacteria: Gram-positive bacteria are common in a pharmaceutical manufacturing environment but not in water systems. This is because they are generally not suited to surviving in a liquid environment that has the chemical purity of a pharmaceutical-grade water system. Gram-positive bacteria include the spore-forming bacteria from the genus *Bacillus*, which are common soil and dust ▲microorganisms,▲1S (USP41) and the non-sporulating bacteria from the genera *Staphylococcus*, *Streptococcus*, and *Micrococcus*, which normally colonize human skin and mucous membranes. Other types of Gram-positive bacterial ▲microorganisms▲1S (USP41) include organisms from the genera *Corynebacterium*, *Mycobacterium*, *Arthrobacter*,

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Propionibacterium, *Streptomyces*, and *Actinomyces*. This latter group of microbes can be found in various natural habitats including the human skin and soil.

Although Gram-positive bacteria can be detected in pharmaceutical water samples, their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. Although these non-aquatic microorganisms could be present in source water and could, in rare circumstances, make their way into the early stages of a water purification unit operation, Gram-positive bacteria are not known to colonize water systems. In addition, these microbes will likely be removed by one or more of the purification unit operations prior to the ultimate creation of the pharmaceutical-grade water.

8.1.2.2 Gram-negative bacteria: These types of bacteria are found in soil, water, plants, and animals. Gram-negative bacteria are ▲relevant▲ 1S (USP41) to pharmaceutical manufacturers, primarily due to their production of endotoxins ▲as well as their ability to populate water systems,▲ 1S (USP41) a topic discussed in *8.4 Endotoxin*. Some Gram-negative bacteria prefer aquatic habitats and tend to colonize water systems and other wet environments as biofilms, a topic discussed in *8.2 Biofilm Formation in Water Systems*.

8.1.2.3 Mycoplasma: Organisms from the genus Mycoplasma are the smallest of the bacteria. Unlike other bacteria, these organisms do not have a cell wall and many exist as intracellular or animal▲/plant▲ 1S (USP41) parasites. Mycoplasmas also ▲may▲ 1S (USP41) require specific nutrients for survival, including ▲sterols,▲ 1S (USP41) and they cannot survive in a hypotonic environment such as pure water. Based on these facts, this type of bacteria is not a concern for pharmaceutical-grade water systems.

8.1.3 FUNGI

Fungi are mainly aerobic mesophilic microbes. They exist as unicellular (yeast) and multicellular filamentous (mold) organisms. Molds are often found in wet/moist but usually non-aquatic environments, such as soil and decaying vegetation. ▲Yeasts are often associated with humans and vegetation, and both yeasts and molds also can be found in pharmaceutical environment.▲ 1S (USP41) As mold matures it develops spores, which, unlike bacterial spores, are part of its reproductive cycle and are less resistant to adverse conditions. Mold spores are easily spread through air and materials, and could contaminate water samples.

▲▲ 1S (USP41)

Neither yeasts nor molds are suited for colonization or survival in pharmaceutical water systems. Their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. These non-aquatic microorganisms, if present in source water, could make their way into the early stages of a water purification system; however, they will likely be removed by one or more of the purification unit operations.

8.1.4 VIRUSES

A virus is a small infectious agent unlike eukaryotes and prokaryotes. This is because viruses have no metabolic abilities of their own. Viruses are genetic elements containing either DNA or RNA that replicate within host cells. Human pathogenic viruses, especially those of fecal origin, could be present in source water. However, they are easily neutralized by typical water purification treatments, such as chlorination. Therefore, it is unlikely that ▲human pathogenic▲ 1S (USP41) viruses will be present or will proliferate (due to the absence of host cells) in pharmaceutical-grade waters.

8.1.5 THERMOPHILES

Thermophiles are heat-loving organisms and can be either bacteria or molds. Thermophilic and hyperthermophilic aquatic microorganisms (see *8.1.1 Archaeans*) require unique environmental and nutritional conditions to survive ▲(e.g., presence of specific inorganic or organic nutrients and their concentrations, extreme pH, presence or absence of oxygen).▲ 1S (USP41) These conditions do not exist in the high-purity water of pharmaceutical water systems, whether ambient or hot, to support their growth. Bacteria that are able to inhabit hot pharmaceutical water systems are invariably found in much cooler locations within these hot systems; for example, within infrequently used outlets, ambient subloops off of hot loops, use-point and sub-loop cooling heat exchangers, transfer hoses and connecting pipes, or dead legs. These bacterial contaminants are the same mesophilic (moderate temperature-loving) types found in ambient water systems and are not thermophiles. Based on these facts, thermophilic bacteria are not a concern for hot pharmaceutical-grade water systems.

8.2 Biofilm Formation in Water Systems

A biofilm is a three-dimensional structured community of sessile microbial cells embedded in a matrix of extracellular polymeric substances (EPS). Biofilms form when bacteria attach to surfaces in moist environments and produce a slimy, glue-like substance, the EPS matrix, while proliferating at that location. This slimy matrix facilitates biofilm adhesion to surfaces as well as the attachment of additional planktonic cells to form a microbial community.

The EPS matrix of biofilms that colonize water systems also facilitates adsorption and concentration of nutrients from the water and retains the metabolites and waste products produced by the embedded biofilm cells, which can serve as nutrients for other biofilm community members.

This EPS matrix is also largely responsible for biofilm's resistance to chemical sanitizers, which must penetrate completely through the matrix to contact and kill the biofilm cells within the matrix. Heat sanitization approaches do not generally have these EPS matrix penetration difficulties, so they are usually considered superior to chemicals in killing biofilms where materials of construction allow.

The three-dimensional structure of a well-developed biofilm, as well as the biofilm's creation and release of small, motile "pioneer cells" for further colonization, are facilitated through gene expression modulating "quorum sensing" chemicals

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released in tiny amounts by individual biofilm cells and concentrated to a functional level within this same EPS matrix. So, the EPS matrix of biofilms is primarily responsible for the biofilm's success in colonizing and proliferating in very low nutrient-containing high-purity water systems. The EPS matrix also explains the difficulty in killing and/or removing biofilms from water purification and distribution system surfaces.

8.2.1 BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Common microorganisms recovered from water system samples include Gram-negative bacteria from the genera *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Stenotrophomonas*, *Comamonas*, *Methylobacterium*, and many other types of *Pseudomonas*-like organisms known collectively as pseudomonads ▲(members of the family *Pseudomonadaceae*).▲ 1S (USP41) These types of microbes, found in soil and source water, tend to colonize all water system distribution and purification system surfaces including activated carbon beds, deionizing resin beds, RO systems, membrane filtration modules, connecting piping, hoses, and valves. If not controlled, they can compromise the functionality of purification steps in the system and spread downstream, possibly forming biofilms on the distribution system surfaces such as tanks, piping, valves, hoses, and other surfaces, from where they can be sheared or otherwise released into the finished water used in processes and products.

Some of the biofilm pseudomonads are opportunistic human pathogens and may possess resistance to commonly used pharmaceutical product preservatives, particularly when embedded in EPS matrix flocs sheared from water system biofilms. Several pseudomonads are also capable of utilizing a wide variety of carbon sources as nutrients, allowing them to colonize austere, adventitious nutrient environments such as water systems. This nutritional diversity also makes them capable of growing to very high numbers in some pharmaceutical products and raw materials, thus leading to product adulteration and potential risk to patient health. Given that these bacteria are commonly found in aqueous environments, endotoxin control for Water for Injection systems (and some Purified Water systems) through biofilm control becomes critical.

8.2.2 NON-BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Other types of non-pseudomonad Gram-negative bacteria, ▲such as▲ 1S (USP41) the genera *Escherichia*, *Salmonella*, *Shigella*, *Serratia*, *Proteus*, *Enterobacter*, and *Klebsiella*, are used as indicators of fecal contamination. Although some of these bacteria ▲are also plant pathogens, others▲ 1S (USP41) can be human enteric pathogens, ▲and can contaminate potable water supplies.▲ 1S (USP41) These non-pseudomonads are not suited to colonizing or surviving in pharmaceutical water systems owing to the water's chemical purity. In fact, non-pseudomonad enteric bacteria are extremely unlikely contaminants of pharmaceutical water systems unless local sewage and source water controls are not in place. Such controls are required in order to comply with the source water requirements for making USP-grade waters as described in their respective monographs.

8.3 Microorganism Sources

8.3.1 EXOGENOUS CONTAMINATION

Exogenous microbial contamination of bulk pharmaceutical water comes from numerous possible sources, including source water. At a minimum, source water should meet the microbial quality attributes of Drinking Water, which is the absence of fecal coliforms (*E. coli*). A wide variety of other types of microorganisms, chiefly Gram-negative bacteria, may be present in the incoming water. If appropriate steps are not taken to reduce their numbers or eliminate them, these microorganisms may compromise subsequent water purification steps.

Exogenous microbial contamination can also arise from maintenance operations, equipment design, and the process of monitoring, including:

- Unprotected, faulty, or absent vent filters or rupture disks
- Backflow from interconnected equipment
- Non-sanitized distribution system openings for component replacements, inspections, repairs, and expansions
- Inadequate drain air-breaks
- Innate bioburden of activated carbon, ion-exchange resins, regenerant chemicals, and chlorine-neutralizing chemicals
- Inappropriate rinsing water quality after regeneration or sanitization
- Poor sanitization of use points, hard-piped equipment connectors, and other water transfer devices such as hoses
- Deficient techniques for use, sampling, and operation

The exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil, air, or even human origin. The detection of non-aquatic microorganisms may be an indication of sampling or testing contamination or a system component failure, which should trigger investigation and remediation. Sufficient care should be given to sampling, testing, system design, and maintenance to minimize microbial contamination from exogenous sources.

8.3.2 ENDOGENOUS CONTAMINATION

Endogenous sources of microbial contamination can arise from unit operations in a water purification system that is not properly maintained and operated. Microorganisms present in source water may adsorb to carbon bed media, ion-exchange resins, filter membranes, and other equipment surfaces, and initiate the formation of biofilms.

Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system. Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment and to distribution systems.

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Another source of endogenous microbial contamination is the distribution system itself. Microorganisms can colonize pipe surfaces, rough welds, misaligned flanges, valves, and dead legs, where they proliferate and form biofilms. Once formed, biofilms can become a continuous source of microbial contamination, which is very difficult to eradicate. Therefore, biofilm development must be managed by methods such as frequent cleaning and sanitization, as well as process and equipment design.

8.4 Endotoxin

▲Bacterial endotoxin is a lipopolysaccharide (LPS) that is a component of the outer cell membrane of Gram-negative bacteria. Endotoxins may occur as collections of LPS molecules associated with living microorganisms, fragments of dead microorganisms, the EPS matrix surrounding biofilm bacteria, or free molecular clusters or micelles containing many lipopolysaccharide molecules. The monomeric form of the endotoxin molecule does not exist in high-purity water because of the molecule's amphipathic nature.▲ 1S (USP41) Some grades of pharmaceutical waters, such as those used in parenteral applications (e.g., Water for Injection, Water for Hemodialysis, and the sterilized packaged waters made from Water for Injection) strictly limit the amount of endotoxins that may be present because these compounds are pyrogenic.

8.4.1 SOURCES

▲▲ 1S (USP41) Endotoxins may be introduced into the system from the source water or may be released from cell surfaces of bacteria ▲in water system biofilms.▲ 1S (USP41) For example, a spike in endotoxin may occur following sanitization as a result of endotoxin release from killed cells. Endotoxin quantitation in water samples is not a good indicator of the level of biofilm development in a water system because of the multiplicity of endotoxin sources.

8.4.2 REMOVAL AND CONTROL

To control endotoxin levels in water systems, it is important to control all potential sources of contamination with Gram-negative bacteria as well as free endotoxin in the water. Contamination control includes the use of upstream unit operations to reduce bioburden from incoming water, as well as engineering controls (e.g., heat sanitization, equipment design, UV sanitizers, filters, material surface, and flow velocity) to minimize biofilm development on piping surfaces and to reduce re-inoculation of the system with free-floating bacteria.

Endotoxin remediation may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system. Examples of endotoxin removal steps in a water purification train include RO, deionization, ultrafilters, ▲distillation,▲ 1S (USP41) and endotoxin-adsorptive filters.

8.5 Test Methods

Microbes in water systems can be detected as exampled in this section or by methods adapted from *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Microorganisms* ⟨62⟩, or the current edition of *Standard Methods for the Examination of Water and Wastewater* by the American Public Health Association. This section describes classical culture approaches to bioburden testing, with a brief discussion on rapid microbiological methods.

Every water system has a unique microbiome. It is the user's responsibility to perform method validation studies to demonstrate the suitability of the chosen test media and incubation conditions for bioburden recovery. In general, users should select the method that recovers the highest planktonic microbial counts in the shortest time, thus allowing for timely investigations and remediation. Such studies are usually performed before or during system validation. ▲▲ 1S (USP41)

The steady state condition can take months or even years to be achieved, and can be affected by a change in source water quality, changes in finished water purity by using modified or increasingly inefficient purification processes, changes in finished water use patterns and volumes, changes in routine and preventative maintenance or sanitization procedures and frequencies, or any type of system intrusion (e.g., component replacement, removal, or addition).

8.5.1 MICROBIAL ENUMERATION CONSIDERATIONS

Most microbial contaminants in water systems are found primarily as biofilms on surfaces, with only a very small percentage of the microbiome suspended in the water, or planktonic, at any given time. Although it would seem logical to directly monitor biofilm development on surfaces, current technology for surface evaluations in an operating water system makes this impractical in a GMP environment. Therefore, an indirect approach must be used: the detection and enumeration of planktonic microorganisms that have been released from biofilms. This planktonic microbiome will impact the processes or products where the water is used.

The detection and enumeration of the planktonic microbiome can be accomplished by collecting samples from water system outlets. Planktonic organisms are associated with the presence of biofilms as well as free-floating bacteria introduced into the system (pioneer cells), which may eventually form new biofilms. Therefore, by enumerating the microorganisms in water ▲▲ 1S (USP41) samples, the overall state of control over biofilm development can be assessed. This assessment has historically been accomplished with classical cultural techniques, which are viewed as the traditional method. However, nutritional limitations of the growth media may not satisfy growth requirements of organisms present in the water system that originated from a biofilm. As a result, traditional cultural methods may only detect a fraction of the biofilm bacteria present in the water sample. Other options are available, such as rapid microbiological methods.

There is no ideal cultural enumeration method that will detect all microorganisms in a water sample, although some media or incubation temperatures may be better than others. However, from a PC perspective, this limitation is acceptable because it is the relative changes in the trends for water sample microbial counts that indicate the state of PC.

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▲Consideration should also be given to the timeliness of microbial testing after sample collection. The number of detectable organisms in a sample collected in a sterile, scrupulously clean sample container will usually decrease as time passes. The organisms within the sample may die or adhere to the container walls, reducing the number that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of nutrients that could promote microbial growth. Because the number of organisms in the water can change over time after sample collection, it is best to test the samples as soon as possible. If it is not possible to test the sample within 2 h of collection, the sample should be held at refrigerated temperatures (2°–8°) and tested within 24 h. In situations where even 24 h is not possible (such as when using off-site contract laboratories), it is particularly important to qualify the microbiological sample hold times and storage conditions to avoid significant changes in the microbial population during sample storage.▲ 1S (USP41)

8.5.2 THE CLASSICAL CULTURAL APPROACH

Classical cultural approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Cultural approaches are further defined by the type of medium used in combination with the incubation temperature and duration. This combination should be selected according to the monitoring needs of a specific water system and its ability to recover the microorganisms of interest, i.e., those that could have a detrimental effect on the products manufactured or process uses, as well as those that reflect the microbial control status of the system.

8.5.2.1 Growth media: The traditional categorization is that there are two basic forms of media available: "high nutrient" and "low nutrient". Those media traditionally categorized as high-nutrient include Plate Count Agar (TGYA), Soybean Casein Digest Agar (SCDA or TSA), and m-HPC Agar (formerly m-SPC Agar). These media are intended for the general isolation and enumeration of heterotrophic or copiotrophic bacteria. Low-nutrient media, such as R2A Agar and NWRI Agar (HPCA), have a larger variety of nutrients than the high-nutrient media. These low-nutrient media were developed for use with potable water due to their ability to recover a more nutritionally diverse population of microorganisms found in these environments. The use of R2A may not be the best choice for high-purity water systems. Even though high-purity water creates an oligotrophic environment, it has been shown empirically that in many high-purity compendial waters, the microbial count disparity between low- and high-nutrient media is dramatically less to nil, compared to potable water. Nevertheless, using the medium that has been demonstrated ▲as acceptable through comparative media analysis is recommended.▲ 1S (USP41)

8.5.2.2 Incubation conditions: Duration and temperature of incubation are also critical aspects of microbiological testing. Classical compendial methods (e.g., ⟨61⟩) specify the use of high-nutrient media, typically incubated at 30°–35° for NLT 48 h. Given the types of microbes found in many water systems, incubation at lower temperatures (e.g., ranges of 20°–25° or 25°–30°) for longer periods (at least 4 days) could recover higher microbial counts than classical compendial methods. Low-nutrient media typically require longer incubation conditions (at least 5 days) because the lower nutrient concentrations promote slower growth. Even high-nutrient media can sometimes yield higher microbial recovery with longer and cooler incubation conditions.

▲8.5.2.3 Selection of method conditions: The decision to test a particular system using high- or low-nutrient media, higher or lower incubation temperatures, and longer or shorter incubation times should be based on comparative cultivation studies using the native microbiome of the water system. The decision to use media requiring longer incubation periods to recover higher counts also should be balanced with the timeliness of results. Detection of marginally higher counts at the expense of a significantly longer incubation period may not be the best approach for monitoring water systems, particularly when the slow growers are not new species but the same as those recovered within shorter incubation times. Some cultural conditions using low-nutrient media lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. The nature of some of the slow growers and the extended incubation times needed for their development into visible colonies also may lead to those colonies becoming dysgonic and difficult to subculture. That could limit their further characterization, depending on the microbial identification technology used. The selection of method parameters should provide conditions that adequately recover microorganisms from the water system, including those that are objectionable for the intended water use.▲ 1S (USP41)

8.5.3 SUGGESTED CLASSICAL CULTURAL METHODS

▲Example methods are presented in *Table 3*.▲ 1S (USP41)

Table ▲3.▲ 1S (USP41) Example Culture Methods

Drinking Water	Pour plate method or membrane filtration method[a]
	Suggested sample volume: 1.0 mL[b]
	Growth medium: ▲Plate Count Agar▲ 1S (USP41)[c]
	Incubation time: 48–72 h[d]
	Incubation temperature: 30°–35°[e]

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Table ▲3.▲ 1S (USP41) Example Culture Methods *(continued)*

Purified Water	Pour plate method or membrane filtration method[b]
	Suggested sample volume: 1.0 mL for pour plate or ▲up to▲ 1S (USP41) 100 mL for membrane filtration[b]
	Growth medium: ▲Plate Count Agar▲ 1S (USP41)[c]
	Incubation time: 48–72 h[d]
	Incubation temperature: 30°–35°[e]
Water for Injection	Membrane filtration method[a]
	Suggested sample volume: 200 mL[b]
	Growth medium: ▲Plate Count Agar▲ 1S (USP41)[c]
	Incubation time: 48–72 h[d]
	Incubation temperature: 30°–35°[e]

[a] A membrane filter with a rating of 0.45 µm is generally considered preferable to smaller porosity membranes.
[b] Sample size must be appropriate for the expected microbial count of the water in order to derive statistically valid colony counts.
[c] For optimum recovery, an alternative medium may be more appropriate (e.g., m-HPC, TSA/SCDA, R2A).
[d] For optimum recovery, alternative incubation times may be needed.
[e] For optimum recovery, alternative incubation temperatures may be needed.

For media growth promotion, use at a minimum *Pseudomonas aeruginosa* ATCC 9027 and *Bacillus subtilis* ATCC 6633. Additional organisms should be used to represent those that are considered objectionable and/or typically isolated from the water system (house isolates).

8.5.4 MICROBIAL IDENTIFICATION

In addition to the enumeration of the bioburden in the water, there is a need to identify and/or select certain microbial species that could be detrimental to products or processes. Some bacteria may also be resistant to preservatives and other antimicrobial chemicals used in ▲nonsterile▲ 1S (USP41) liquid and semi-solid products, thus leading to potential product spoilage. For example, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, as well as some other pseudomonads, are known opportunistic pathogens ▲under certain conditions.▲ 1S (USP41) As such, it may be appropriate to consider these species as objectionable microorganisms for the type of water used to manufacture ▲nonsterile▲ 1S (USP41) liquid and semi-solid products. There is a higher risk of infection if these organisms are found in products targeted for susceptible patient populations (e.g., the very young, the very old, and the immunocompromised) or products contacting highly susceptible tissues (e.g., inhaled products or some topical products). However, if the product where the water is used carries an absence specification for a particular pathogenic species that is not capable of living in a high-purity water system ▲(e.g., *Staphylococcus aureus* or *Escherichia coli*),▲ 1S (USP41) then these non-aquatic species should not be candidates for routine ▲recovery testing from water samples.▲ 1S (USP41) For more information, see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩, *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩, and *Microbiological Best Laboratory Practices* ⟨1117⟩.

For PC and QC, it is valuable to know the microbial species present in the normal microbiome of a water system, even if they are not specifically objectionable. If a new species is detected, it may be an indication of a subtle process change or an exogenous intrusion. The identity of the microorganism may be a clue as to its origin and can help with implementation of corrective or preventive action. Therefore, it is industry practice to identify the microorganisms in samples that yield results exceeding established Alert and Action Levels. It is also of value to periodically identify the normal microbiome in a water system, even if counts are below established Alert Levels. This information can provide perspective on the species recoveries from Alert and Action Level excursion samples, indicating whether they are new species or just higher levels of the normal microbiome. Water system isolates may be incorporated into a company culture collection for use in tests such as antimicrobial effectiveness tests, microbial method validation/suitability testing, and media growth promotion. The decision to use ▲water isolates in these studies should be risk-based because many such isolates may not grow well on the high-nutrient media required. And because once adapted to laboratory media, they may not perform like their wild type progenitors.▲ 1S (USP41)

8.5.5 RAPID MICROBIOLOGICAL METHODS

In recent years, new technologies that enhance microbial detection and the timeliness of test results have been adopted by pharmaceutical QC testing labs. Rapid Microbiological Methods (RMM) are divided into four categories: Growth-Based, Viability-Based, ▲Metabolite-Based,▲ 1S (USP41) and Nucleic Acid-Based. Examples of RMM used for the evaluation of microbial quality of water systems include:

- Microscopic visual epifluorescence membrane counting techniques
- Automated laser scanning membrane counting approaches
- Early colony detection methods based on autofluorescence, adenosine triphosphate (ATP) bioluminescence, or vital staining
- Genetic-based detection/quantitation

See *Validation of Alternative Microbiological Methods* ⟨1223⟩ for further information on rapid microbiological methods.

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9. ALERT AND ACTION LEVELS AND SPECIFICATIONS

9.1 Introduction

Establishment of Alert and Action Levels for any manufacturing process facilitates appropriate and timely control. In the case of a pharmaceutical water system, the key PC parameters can be specific chemical, physical, and microbiological attributes of the water produced. Typically, most chemical attributes can be determined in real time or in the lab within a few minutes after sample collection. Physical attributes such as the pressure drop across a filter, temperature, and flow rate—which are sometimes considered critical for operation or sanitization of the water system—must be measured in situ during operation. Obtaining timely microbial data is more challenging compared to chemical and physical attributes, often taking several days. This limits the ability to control microbial attributes in a timely manner, and therefore requires a more challenging evaluation of the test results and conservative implementation of PC levels. This section provides guidance on the establishment and use of Alert and Action Levels, as well as Specifications to assess the suitability of the water and the water system for use in production.

9.2 Examples of Critical Parameter Measurements

Examples of measurements and parameters that are important to water system processes and products are described below. The list, which is not intended to be exhaustive or required, contains some examples of parameters that could be measured to demonstrate that the system is in a state of control.

Examples of measurements that could be critical to the purification or sanitization process include:

- Temperature, for thermally sanitized systems
- Percent rejection of an RO system
- Endotoxin levels of feed water to a distillation system
- Chlorine presence immediately prior to an RO system

Examples of measurements that could be critical to the water distribution process include:

- Return/end-of-loop line pressure, to forewarn of ▲the potential to aspirate air or fluids because of▲ 1S (USP41) simultaneous use of too many outlets
- ▲Temperature to assure the self-sanitizing conditions are maintained for a hot water system▲ 1S (USP41)
- Flow rate, to ensure that sufficient water is available for operations

Examples of measurements that could be critical to final water quality include:

- Conductivity
- TOC
- Endotoxin—for Water for Injection systems
- ▲ ▲ 1S (USP41)
- Bioburden
- Ozone or other chemicals—for chemically sanitized systems

9.3 Purpose of the Measurements

Although the purpose of each measurement varies, the results can be used to provide system performance feedback, often immediately, serving as ongoing PC and product quality indicators. At the same time, the results provide information necessary for making decisions regarding the immediate processing and usability of the water (see *6.1 Purposes and Procedures*). However, some attributes may not be monitored continuously or may have a long delay in data availability (e.g., microbial data). Regardless, both real-time data and data with longer cycle times can be used to properly establish Alert and Action Levels, which can serve as an early warning or indication of a potentially approaching quality shift.

As PC indicators, Alert and Action Levels are trigger points for the potential need for investigation and/or remedial action, to prevent a system from deviating from normal conditions and producing water unsuitable for its intended use. This "intended use" minimum quality is sometimes referred to as a "Specification" or "Limit", and may include limits for conductivity and TOC listed in water monographs, or other specifications required for these waters that have been defined by the user internally.

In all cases, the validity of the data should be verified to ensure that the data are accurate and consistently representative of the water quality in the system, regardless of whether the sample was collected from a sampling port or use point. The resulting data must not be unduly biased, positively or negatively, due to the sampling method, the environment in the vicinity of the sampling location, the test procedure, instrumentation, or other artifacts that could obscure or misrepresent the true quality of the water intended by the purpose of the sampling, i.e., for PC or for QC.

9.4 Defining Alert and Action Levels and Specifications

Data generated from routine water system monitoring should be trended to ensure that the system operates in a state of chemical and microbiological control. To assist with the evaluation of system performance, companies should establish in-process control levels based on historical data or a fraction of the water Specifications (as long as this latter approach yields values with relevance to process performance).

When establishing Alert and Action Levels and Specifications, a two- or three-tier approach is typically used. In a three-tier approach, the typical structure is to establish in-process controls using "Alert Level", "Action Level", and "Specifications". Alert

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and Action Levels are used as proactive approaches to system management prior to exceeding Specifications. The criteria for defining and reacting to adverse trends should be set by the user. These levels should be set at values that allow companies to take action to prevent the system from producing water that is unfit for use. Water Specifications or Limits represent the suitability for use of the water.

In a two-tier approach, a combination of the above terminology is used, depending on the parameter to be monitored. For example, if the attribute does have a monograph specification, the two tiers are Alert Level (or Action Level) and Specification. If the attribute does not have a limit/specification, the two tiers are usually Alert Level and Action Level.

A single-tier approach is possible, but this is risky and difficult to manage. With this approach, where the water/system is either acceptable or not acceptable, the single-tier method does not allow for any adjustment, correction, or investigation prior to stopping production.

However, certain sampling locations, such as sampling ports that are not used for manufacturing products or processes, do not represent the finished water quality where a Specification could be applied. In these locations, a two-tier approach (Alert and Action Levels only) could be applied. In some sampling locations, a single PC level might possibly be appropriate, depending on the attribute.

9.4.1 ALERT LEVEL

An Alert Level for a measurement or parameter should be derived from the normal operating range of the water system. Specifically, Alert Levels are based on the historical operating performance under production conditions, and then are established at levels that are just beyond the majority of the normal historical data. The Alert Level for a parameter is often a single value or a range of values, such as:

- Higher than typical conductivity or TOC
- Higher than typical microbial count
- Higher than typical endotoxin level
- Low temperature during thermal sanitization
- pH range control prior to an RO
- Ozone concentration in a storage tank

Various methods, tools, and statistical approaches are available for establishing Alert Levels, and the user needs to determine the approaches that work for their application. Some numerical examples are two or three standard deviations ▲▲1S (USP41)(or more) in excess of the mean value, or some percentage above the mean value but below a Specification. An event-based example could be the appearance of a new microorganism or a non-zero microbial count where zero is the norm.

When an Alert Level is exceeded, this indicates that a process or product may have drifted from its normal operating condition or range. Alert Level excursions represent a warning and do not necessarily require a corrective action. However, Alert Level excursions may warrant notification of personnel involved in water system operation, as well as the quality assurance (QA) personnel. Alert Level excursions may also lead to additional monitoring, with more intense scrutiny of the resulting and neighboring data as well as other process indicators.

9.4.2 ACTION LEVEL

An Action Level is also based on the same historical data, but the levels are established at values (or ranges) that exceed the Alert Levels. The values/ranges are determined using the same types of numerical or event-based tools as the Alert Levels, but at different values▲/ranges.▲ 1S (USP41)

In a three-tier approach, it is good practice to select an Action Level that is more than the Alert Level, but less than the Specification to allow the user to make corrective actions before the water would go out of compliance.

Exceeding a quantitative Action Level indicates that the process has allowed the product quality or other critical parameter to drift outside of its normal operating range. An Action Level can also be event-based. In addition to exceeding quantitative Action Levels, some examples of event-based Action Level excursions include, but are not limited to:

- Exceeding an Alert Level repeatedly
- Exceeding an Alert Level in multiple locations simultaneously
- The recovery of specific objectionable microorganisms
- A repeating non-zero microbial count where zero is the norm

If an Action Level is exceeded, this should prompt immediate notification of both QA staff and the personnel involved in water system operations and use, so that corrective actions can be taken to restore the system back to its normal operating range. Such remedial actions should also include investigative efforts to understand what happened and eliminate or reduce the probability of recurrence. Depending on the nature of the Action Level excursion, it may be necessary to evaluate its impact on the water uses during the period between the previous acceptable test result and the next acceptable test result.

9.4.3 SPECIAL ALERT AND ACTION LEVEL SITUATIONS

In new or significantly altered water systems, where there is limited or no historical data from which to derive trends, it is common to establish initial Alert and Action Levels based on equipment design capabilities. These initial levels should be within the process and product Specifications where water is used. It is also common for new water systems, especially ambient water systems, to undergo changes, both chemically and microbiologically, over time as various unit operations (such as RO membranes) exhibit the effects of aging. This type of system aging effect is most common during the first year of use. As the system ages, a steady state ▲microbiome▲ 1S (USP41) (microorganism types and levels) may develop due to the collective effects of system design, source water, maintenance, and operation, including the frequency of re-bedding, backwashing, regeneration, and sanitization. This established or mature ▲microbiome▲ 1S (USP41) may be higher than the one detected when

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the water system was new. Therefore, there is cause for the impurity levels to increase over this maturation period and eventually stabilize.

Some water systems are so well controlled microbially—such as continuously or intermittently hot Water for Injection distribution systems—that microbial counts and endotoxin levels are essentially nil or below the limit of reasonable detectability. This common scenario often coincides with a very low Specification that is poorly quantifiable due to imprecision (as much as two-fold variability) of the test methods that may be near their limits of detection. In such systems, quantitative data trending has little value, and therefore, quantitative PC levels also have little value. The non-zero values in such systems could be due to sporadic sampling issues and not indicative of a water system PC deviation; however, if these non-zero values occur repeatedly, they could be indicative of process problems. So, an alternative approach for establishing Alert and Action Levels with these data could be the use of the incident rate of non-zero values, with the occasional single non-zero "hit" perhaps being an Alert Level (regardless of its quantitative value), and multiple or sequential "hits" being an Action Level. Depending on the attribute, perhaps single hits may not even warrant being considered an Alert Level, so only a multiple-hit situation would be considered actionable. It is up to the user to decide on their approach for system control, i.e., whether to use one, two, or three levels of controls for a given water system and sampling location, and whether to establish Alert and Action Levels as quantitative or qualitative hit-frequency values.

9.4.4 SPECIFICATIONS

Water Specifications or Limits are set based on direct potential product and/or process impact and they represent the suitability for use of the water. The various bulk water monographs contain tests for *Conductivity*, *TOC*, and *Bacterial Endotoxins* (for *Water for Injection*). Aside from the monographs for *Water for Hemodialysis* and multiple sterile waters, microbial specifications for the bulk waters are intentionally not included in their monograph tests.

The need for microbial specifications for bulk waters (*Purified Water* and *Water for Injection*) depends on the water use(s), some of which may require strict control (e.g., very low bioburden, absence of objectionable organisms, or low ionic strength) while others may require no specification due to the lack of impact. For example, microbial specifications are appropriate and typically expected for water that is used in product formulations and final equipment rinses. ▲Where the water is used for analytical reagent preparations and the analytical method is not affected by microbial contaminants,▲ 1S (USP41) or for cleaning processes that conclude with a final antimicrobial heat drying or solvent rinsing step, the microbial quality of the water is likely less of a concern. The decision to establish microbial Specifications for bulk pharmaceutical waters should be based on a formal risk assessment of its uses and justified by scientific rationale.

It is very important to understand the chemical and microbial quality of the water in its final form as it is delivered from a water system to the locations where it is used in manufacturing activities and other points of use. The quality of the water within the water system could be compromised if it picks up chemical or microbial contaminants during its delivery from the system to the points of use. These points of use, where cumulative contamination could be present, are the locations where compliance with all the water Specifications is mandated.

As discussed above, compliance with chemical Specifications can be confirmed periodically between uses, immediately prior to use, or even while the water is being utilized in product manufacturing. While the use of RMM may provide for timely microbial data, the use of conventional cultivative microbiological testing usually delays confirmation of microbial compliance until after the water has been used. However, for some applications, this logistical limitation should not eliminate the need for establishing microbial Specifications for this very important raw material.

The manufacturing risk imposed by these logistics accentuates the value of validated microbial control for a water system. It also emphasizes the value of ▲unbiased sampling for microbial monitoring (e.g., influences from technique, hoses, flushing)▲ 1S (USP41) of samples collected from pertinent locations, with evaluation of the resulting data against well-chosen, preferably trend-derived Alert and Action Levels, which can facilitate remedial PC to preclude Specification excursions.

Users should establish their own quantitative microbial Specifications suited to their water uses. But these values should not be greater than 100 cfu/mL for *Purified Water* or 10 cfu/100 mL for *Water for Injection* unless specifically justified, because these values generally represent the highest microbial levels for pharmaceutical water that are still suitable for manufacturing use.

A Specification excursion should prompt an out-of-specification (OOS) investigation. The investigation is performed to determine 1) the root cause of the excursion so that CAPA may be taken for remediation purposes, and 2) assess the impact on affected processes and finished products where the water was used. Product disposition decisions must be made and are dependent on factors that could include:

- Role of water in the product or in-process material
- Chemical or microbial nature of the attribute whose Specification value was exceeded
- Level of product contamination by the water
- Presence of objectionable microorganisms
- Any downstream processing of affected in-process materials that could mitigate the OOS attribute
- Physical and chemical properties of the finished product where the water was used that could mitigate the OOS attribute
- Product administration routes and potentially sensitive/susceptible users

9.4.5 SOURCE WATER CONTROL

The chemical and microbial attributes of the starting source water are important to the ability of the water system to remove or reduce these impurities to meet the finished water Specifications (see *2. Source Water Considerations*). Using the example microbial enumeration methods in *Table* ▲3▲ 1S (USP41) a reasonable maximum bacterial Action Level for source water is 500 cfu/mL. This number is derived from U.S. EPA NPDWR where it is used as an Action Level for the water authority indicating the need for improving disinfection and water filtration to avoid the penetration of viral, bacterial, and protozoal pathogens into

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the finished Drinking Water. It is not, however, a U.S. EPA heterotrophic plate count Specification or Maximum Contaminant Level (MCL) for Drinking Water.

Nevertheless, of particular importance could be the microbial and chemical quality of this starting water because the water is often delivered to the facility at a great distance from its source and in a condition over which the user has little or no control. High microbial and chemical levels in source water may indicate a municipal potable water system upset, a change in the supply or original water source, a broken water main, or inadequate disinfection, and therefore, potentially contaminated water with objectionable or new microorganisms or coincidental chemical contaminants.

Considering the potential concern about objectionable microorganisms and chemical contaminants in the source water, contacting the water provider about the problem should be an immediate first step. In-house remedial actions could also be needed, including performance of additional testing on the incoming water (as well as the finished water in some cases) or pretreating the water with additional microbial and chemical purification operations (see *5.1 Unit Operations Considerations*).

⟨1234⟩ VACCINES FOR HUMAN USE—POLYSACCHARIDE AND GLYCOCONJUGATE VACCINES

INTRODUCTION

This chapter describes best practices for production, conjugation, and characterization of polysaccharide and glycoconjugate vaccines. It describes key quality attributes at each step of the process and suggests best methods to assess these attributes. The scope of this chapter includes vaccines consisting of one or more purified polysaccharides (such as pneumococcal, meningococcal, and Typhoid Vi vaccines) and components involved in their production, and vaccines consisting of one or more glycoconjugate immunogens in which a saccharide has been covalently attached to a suitable carrier protein. The latter category includes *Haemophilus influenzae* type b (Hib), meningococcal, and pneumococcal conjugate vaccines. The chapter does not include combination vaccines in which Hib conjugates are combined with unrelated immunogens against diphtheria, tetanus, and pertussis.

BACKGROUND

Occurrence of Capsular Polysaccharides

Many pathogenic bacteria possess a polysaccharide capsule that encloses the cell, modulates the flow of nutrients to the cell surface, and protects against dehydration. When a bacterium establishes an infection in a mammalian host, the polysaccharide capsule hides cell surface components from elements of the mammalian immune system, such as antibodies and complement proteins that otherwise would activate mechanisms to kill the pathogen. Although polysaccharide capsules are themselves immunogenic in children and adults, development of a protective immune response may be too slow to defend the host against disease. In many cases, antibodies directed against the capsular polysaccharide are protective, and the prior existence of these antibodies prevents establishment of the infection; this is the basis for their use as vaccines or components of conjugate vaccines. Many bacterial species can be divided into serogroups or serotypes that express structurally and immunologically distinct capsular polysaccharides. The number of known serotypes differs between organisms. There are six known *Haemophilus influenzae* serotypes and more than 90 *Streptococcus pneumoniae* serotypes. Different serotypes (or serogroups) of the same organism may have different infectivities. For instance, the large majority of disease caused by *H. influenzae* is due to the b serotype. Different serotypes may cause disease in different geographical regions, may produce a different spectrum of disease, or may be prevalent in different age groups. The serotype-specific pattern of disease may change with time within a given geographical region. For these reasons, many polysaccharide and conjugate vaccines contain multiple saccharide immunogens.

Structures of Capsular Polysaccharides

Capsular polysaccharides are high molecular weight polymers that contain a strict repeat unit. This repeat unit can be a single monosaccharide unit or can be an oligosaccharide unit that contains as many as eight sugar residues. The repeat units can be linear or branched, and are sometimes linked together by phosphodiester bonds. Bacterial polysaccharides often contain unusual sugar residues that are not found elsewhere, and may be substituted with a wide range of acylating groups (*O*-acetyl groups are the most common) and phosphorylated substituents such as phosphoglycerol. Incomplete *O*-acetylation and migration of *O*-acetyl groups between different hydroxyl groups leads to a degree of heterogeneity in the polysaccharides.

Immune Responses to Capsular Polysaccharides

Capsular polysaccharides are T-cell–independent type 2 immunogens. These immunogens do not evoke antibody isotype switching, affinity maturation, or immunological memory. Because of the relatively late development of the relevant arm of the human immune system, unconjugated polysaccharide vaccines usually induce only a poor immune response in infants under the age of two and thus are not used for this population group. Adjuvants in polysaccharide vaccines do not improve the immune response. In a typical adult target population only a single dose is required to induce a protective immune response. In the absence of immunological memory, regular revaccination is required, often at five-year intervals. Repeated vaccination can lead to hyporesponsiveness to the vaccine.

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Conjugation of Polysaccharides to Carrier Protein

Covalent attachment of a capsular polysaccharide, or an oligosaccharide derived from it, to a protein carrier creates a conjugate vaccine. Immunization with a conjugate vaccine induces humoral immunity by means of a different molecular mechanism that does not require cross-linking of immune cell surface proteins. For this reason, effective conjugate vaccines can be produced using oligosaccharide haptens that may be derived from a capsular polysaccharide or from inherently lower molecular weight polysaccharides such as the *O*-chain of a lipopolysaccharide. Conjugates can also be produced using naturally high-mass polysaccharides or by controlled size reduction to shorter chains. Depending on the manufacturing process there are three basic structural models for conjugate vaccines:

- conjugates in which a carrier protein is modified with multiple oligosaccharide chains that have one or two activation sites to allow attachment to a carrier protein, resulting in a monomeric glycoconjugate or a glycoconjugate with limited cross-linking
- cross-linked conjugates in which multiple activated polysaccharide chains and carrier proteins couple to multiple polysaccharide chains, creating a cross-linked network of proteins and glycans
- conjugates in which a size-reduced polysaccharide is covalently attached to a complex of proteins, typically bacterial outer proteins, via multiple attachments.

The Carrier Protein

The most widely used carrier proteins are related to bacterial toxins that are detoxified by chemical or genetic means. Conjugate vaccines induce a T-cell–dependent response that is developed early in life and leads to immunological memory and boosting of the response by further doses of the vaccine, thus they are suitable for infant immunization. The role of the carrier protein in modulating the immune response is discussed below.

Conjugation Chemistry

Polysaccharides can be covalently attached to proteins, although activation of the polysaccharide is required. Sometimes the carrier protein is also activated to create compatible reactive groups. Polysaccharides can be activated initially by creation of reactive aldehyde groups, by periodate oxidation or uncovering of the reducing terminal of sugars, by reaction of hydroxyl groups with highly reactive reagents such as cyanogen bromide (or 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate [CDAP] as a crystalline alternative) or carbonyldiimidazole, by use of carboxylic acid groups, or, if available, by free amino groups or phosphate groups. Periodate oxidation, dilute acid hydrolysis, and some other approaches to polysaccharide activation may, depending on the structure of the polysaccharide, lead to depolymerization of oligosaccharides, which are typically fractionated by size and an appropriate fraction coupled to the carrier. The "natural" reactive groups on proteins include lysine ε-amino groups, *N*-terminal amino groups, carboxylic acids in aspartate or glutamate, or thiol groups. Carrier protein activation may also involve the creation of hydrazide groups or thiols for linkage. Covalent attachment between the activated polysaccharide and the carrier protein, activated or not, can take place via the use of activated esters, sometimes created *in situ* with a water-soluble carbodiimide reagent, by reduction of Schiff's bases, by reaction of a thiol with maleimide, or by elimination of bromide. Conjugation can either be direct, as in reductive amination, or via the introduction of a suitable bifunctional linker. In general, the choice of conjugation chemistry is defined by either the structure of the polysaccharide repeat unit or the desire to produce a conjugate of a specific structural family.

Immune Responses to Conjugate Vaccines

The immune response to the saccharide component of conjugate vaccines is T-cell–dependent and is similar to the response for proteins, although this process is still not fully understood with respect to glycoconjugates. Because this immunological pathway is in place even in infants, conjugate vaccines were developed initially for use in infants. Following interaction with antigen-presenting cells such as dendritic cells, macrophages, and B-cells, glycoconjugate vaccines are internalized and processed into small peptides and glycopeptides that then are re-exposed and presented to T-lymphocytes in association with the major histocompatibility complex class II molecules. Multiple immunizations of an immunologically naive infant are normally required to raise an antibody response, but processes such as isotype switching and affinity maturation take place and immunological memory is stimulated. In general terms, these processes result in induction of long-lasting high-affinity antibodies that are effective at preventing bacterial pathogens from establishing an infection. Adjuvants in glycoconjugate vaccines are effective at boosting immune responses. These vaccines have also been shown, in the cases of Hib, meningococci, and pneumococci, to eliminate nasopharyngeal carriage of organisms, thus an important aspect of their effectiveness arises from suppressing transmission of infectious serotypes between individuals, called a *herd effect*.

KEY QUALITY PARAMETERS FOR BULK POLYSACCHARIDES

Bulk monovalent polysaccharide is purified from bacterial cell culture and is a key stable intermediate in the manufacture of both polysaccharide and conjugate vaccines. Many quality parameters for final vaccines can be initially assessed by analysis of bulk monovalent polysaccharides or by using critical in-process tests supporting the manufacturing process. Important quality parameters for purified polysaccharide include identity, purity, composition, and molecular size, and depend on the type and extent of further processing. The purification process is validated to consistently produce compliant material.

The purity of the polysaccharides depends on the purification steps, including harvest methods, clarification, and downstream purification processes. For purification, a combination of precipitation, filtration, and chromatographic procedures can be used, depending on the chemical nature of the polysaccharide. The final purification step can consist of buffer exchange and filtration

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followed by storage of purified polysaccharide (frozen), or additional precipitation and washing of the precipitate with solvent before drying, followed by storage. Drying of polysaccharides can be performed in dessicators and can include several steps of grinding or fluffing and return to the dessicators for further drying. Manufacturers should take care during these steps because mechanical handling of the polysaccharide can reduce its molecular size. The purified polysaccharide is stored at a suitable temperature in conditions that avoid the uptake of moisture. Lyophilization of polysaccharides is also possible. The stability of the polysaccharide under specified storage conditions should be demonstrated; this may include assessment of the optimal moisture content of the dried material.

Differentiating which tests should be used for polysaccharide release and which are better suited to in-process testing to ensure process consistency depends on the process and how the polysaccharide will be further processed. Final decisions about process parameters typically take place after discussions with regulatory authorities.

Dry Weight

Because dry weight is used both to calculate the results of certain tests with reference to the dried substance and to calculate amounts for subsequent processes, volatile matter, including water, in the purified polysaccharide is determined by a combination of suitable methods including the following:

- thermogravimetry (see *Loss on Drying* ⟨731⟩)
- Karl Fischer (water only: see *Water Determination* ⟨921⟩)
- residual solvents determined by gas chromatography (see *Residual Solvents* ⟨467⟩ and *Chromatography* ⟨621⟩) and by nuclear magnetic resonance (NMR) spectroscopy (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩) or colorimetric methods.

Polysaccharide Identity

The manufacturer is required to confirm the identity of the active component present in the purified polysaccharide. If other polysaccharides are produced at the same manufacturing site, the method should be validated to show that it distinguishes the desired polysaccharide from all other polysaccharides produced in that facility (see 21 CFR 610.14). The identity is determined by a prespecified combination of suitable methods such as:

- Immunological test methods: These methods require access to highly specific antisera that are able to distinguish between closely related polysaccharide antigens. Commonly used formats for this purpose are immunoprecipitation, immunoelectrophoresis, and enzyme-linked immunosorbent assays (ELISA). The (serological) specificity of the antiserum should be demonstrated by the absence of cross-reactivity with heterologous polysaccharides manufactured in the same facility. More information regarding these methods can be found in *Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Enzyme–Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and *Immunological Test Methods —Immunoblot Analysis* ⟨1104⟩.
- NMR spectroscopy (see ⟨761⟩): NMR methods require access either to authentic samples of the polysaccharide or to reference spectra. Comparison is made visually in terms of the positions, relative intensities, and multiplicities of significant resonances, or by use of other objective methods.
- Polysaccharide identity can also be assessed by use of a matrix of compositional assays, usually with colorimetric or chromatographic readouts, that define factors such as the amounts of different sugar types (e.g., amino sugars, uronic acids, or methyl pentoses depending on the polysaccharide), the amount of substituents (e.g., *O*-acetyl groups), and the content of nitrogen and phosphorus.

Polysaccharide Purity and Quantity

COLORIMETRIC ASSAYS

Historically, polysaccharide content compared to dry weight (purity) was estimated using a range of colorimetric assays for functional groups specific to the polysaccharide antigen. Because the reference standard is typically a pure monosaccharide or similar model compound, and because assays were typically determined before most structures were known, component quantification may not parallel true stoichiometry. Thus, manufacturers should develop correlations among alternative approaches. Purity can be calculated based on the method employed and the salt form present. *Table 1* lists colorimetric tests that may be appropriate to determine the composition of a particular polysaccharide within a vaccine. The response factors for the sugar units in the polysaccharide may differ from those of a pure monosaccharide reference standard. Manufacturers should address these concerns during method validation.

Table 1. Colorimetric Assays for Polysaccharide Composition and Quantity

	Orcinol Assay (Bial Reagent)	Molybdate (Chen) Assay	Resorcinol Assay (Seliwanoff Reagent)	Sulfuric Acid Digestion	Carbazole Assay	Dimethylaminobenzaldehyde Assay	Cysteine Sulfuric Acid Assay	Hestrin Assay[a]	Anthrone-Sulfuric Acid Assay
PS Antigen	Ribose	Phosphate	Sialic Acid	Total Nitrogen	Uronic Acids	Hexosamines	Methylpentoses	*O*-Acetyl	Total Sugar
Hib PRP	X	X							
MenA		X						X	

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Table 1. Colorimetric Assays for Polysaccharide Composition and Quantity *(continued)*

	Orcinol Assay (Bial Reagent)	Molybdate (Chen) Assay	Resorcinol Assay (Seliwanoff Reagent)	Sulfuric Acid Digestion	Carbazole Assay	Dimethylaminobenzaldehyde Assay	Cysteine Sulfuric Acid Assay	Hestrin Assay[a]	Anthrone-Sulfuric Acid Assay
PS Antigen	Ribose	Phosphate	Sialic Acid	Total Nitrogen	Uronic Acids	Hexosamines	Methylpentoses	*O*-Acetyl	Total Sugar
MenC			X					X	
MenY			X					X	X
MenW135			X					X	X
Pneumo (Serotype Specific)		X	X	X	X	X	X	X	X
Vi								X	

[a] The hestrin assay is appropriate for compositional analysis and identity, but because the degree of *O*-acetylation can vary between polysaccharide batches it is not normally a suitable assay for polysaccharide quantitation.

HYDROLYSIS AND COMPOSITION ANALYSIS BY CHROMATOGRAPHY

Acid or base hydrolysis depolymerizes polysaccharides into oligosaccharides, monosaccharides, or smaller fragments that are polysaccharide-specific for the optimized hydrolysis conditions employed. Aggressive hydrolysis conditions can destroy some components of the polysaccharide. These fragments can be quantified directly by use of, for example, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) or conductivity detection for ions (HPAEC-CD), or by reversed phase high-performance liquid chromatography (HPLC) of fluorophore-labeled sugars. Alternatively, the hydrolysate can be derivatized and analyzed by gas chromatography with flame ionization (GC-FID) or mass spectrometric detection (GC-MS). Suitable polysaccharide reference materials or monosaccharides are required, and the hydrolysis conditions are product-specific. *Table 2* summarizes the type of hydrolysis applied, the analytical methods, and the analytes that have been quantified for different polysaccharides.

Table 2. Chromatographic Methods for Compositional Analysis and Quantification of Vaccine Polysaccharides[a]

PS Antigen	Acid Hydrolysis and HPAEC	Acid Hydrolysis, Fluorophore Labeling, and HPLC	HF Hydrolysis and HPAEC	Base Hydrolysis and HPAEC	Methanolysis and GC or HPAEC
PRP	Ribitol	—	Phosphate	PRP monomer	—
MenA	ManN-6-P	—	Phosphate	*O*-acetyl	—
MenC	Neu5Ac	—	—	*O*-acetyl	—
MenY	Glc, Neu5Ac	Neu5Ac	—	*O*-acetyl	—
MenW135	Gal, Neu5Ac	Neu5Ac	—	*O*-acetyl	—
Pneumo (Serotype Specific)	Alditols, methylpentoses, hexoses, hexosamines, uronic acids, Pyruvate	—	Phosphate	*O*-acetyl	Alditols, methylpentoses, hexoses, hexosamines, uronic acids
Vi	—	—	—	*O*-acetyl Vi monomer fragment	—

[a] PS = polysaccharide; HF = hydrofluoric acid; PRP = polyribosylribitol phosphate; MenA = Meningococcus group A; MenC = Meningococcus group C; MenY = Meningococcus group Y; MenW135 = Meningococcus group W135; Pneumo = Pneumococcus; Man = mannose; Neu = neuraminic acid; Ac = acetyl; Glc = glucosamine; Gal = galactose.

CAPILLARY ELECTROPHORESIS

Capillary zone electrophoresis has been used for the identification and quantification of meningococcal polysaccharides without depolymerization.

IMMUNOCHEMICAL ASSAYS

Immunochemical assays such as immunonephelometry or ELISA require access to specific antisera that must be calibrated to reference materials. The response may be modified by the matrix or size of the polysaccharide.

NMR SPECTROSCOPY

The relative intensities of characteristic resonances can confirm the proportions of different sugar residue types and substituents such as *N*- or *O*-acetyl or pyruvate that are present in the polysaccharide. Quantification can be achieved by comparison of these intensities to that of an added internal standard (see ⟨761⟩).

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Regarding phosphorus determination, one should remember that a number of bacterial polysaccharides contain phosphodiester linkages. The polysaccharide may be quantified based on its phosphorus content by colorimetric assays or instrumental approaches such as inductively coupled plasma–optical emission (ICP-OES) or ICP-mass spectrometry (ICP-MS).

COUNTERIONS

If the percentage purity of polysaccharide bulks (compared to dry weight) is part of their release program, the amount and type of counterion present must be considered and can be determined by, for example, ICP-MS. This is normally an in-process control step.

Polysaccharide Molecular Size Distribution

The molecular size distribution is generally evaluated by liquid chromatography using soft gel–filtration procedures or size exclusion–high-performance liquid chromatography (SEC-HPLC) equipped with in-line refractive index (RI). The results are reported as the distribution coefficient (K_D) determined from the main peak of the elution curve or as the percentage of material that elutes before a defined K_D cut-off value. The absolute molecular weight of the polysaccharide and its hydrodynamic (gyration) radius can be determined by coupling static light scattering and RI detectors to the SEC-HPLC column, and measuring the RI increment (dn/dc) using reference polysaccharides. Requirements based on molecular weight can be expressed in terms similar to those based on molecular size, and related either to peak values or to the proportion that elutes before a defined cut-off value.

Level of Protein Contamination

The residual protein content of the polysaccharide should be determined by an appropriate assay and should be shown to be below the approved specification. These specifications typically vary by polysaccharide and serotype. Method validation should assess the need for sample pretreatment before protein determination and specific interference by the polysaccharide in the protein assay. Further, method validation should demonstrate that the assay sensitivity is appropriate for the specification. *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩ contains information regarding these assays. Methods that are typically applied to polysaccharides include colorimetric assays (e.g., Lowry, Biuret, bicinchonic acid, or Bradford assays) and UV absorbance.

Level of Nucleic Acid Contamination

The nucleic acid content of the purified polysaccharide should be determined and should be shown to meet specifications, which are typically less than 1% w/w. *Nucleic Acid–Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩ provides additional information about these methods.

Quantification of Process- and Product-Related Impurities

Depending on in-process testing or release requirements, chromatographic and spectroscopic methods can be used to quantify residuals from the fermentation and isolation/purification steps. These residuals may include antifoaming agents, phenol, cetyltrimethyl ammonium bromide, ethanol, and other residual solvents. Other impurities should also be quantified using appropriate tests, including:

- bacterial endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩)
- pyrogens (see *Pyrogen Test* ⟨151⟩)
- sterility (see *Sterility Tests* ⟨71⟩), if required
- bioburden (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩), where appropriate.

As an in-process control, the proportion of pneumococcal C polysaccharide in pneumococcal capsular polysaccharides can be determined by a combination of ^{1}H and ^{31}P NMR spectroscopy or by HPAEC-PAD analysis of ribitol. Alternatively, it can be derived from the compositional analysis.

Stability-Indicating Tests

The polysaccharide may lose integrity because of gradual hydrolysis, resulting in a reduced molecular size, and this degradation can be monitored by size exclusion chromatography or by high-performance size exclusion chromatography (HPSEC) with static light scattering detectors for molecular weight. Loss of *O*-acetyl groups can be monitored by HPLC methods. Both the loss or migration of *O*-acetyl groups and the integrity of phosphate-containing polysaccharides can be tracked by NMR spectroscopy. Immunochemical methods can also be used to monitor polysaccharide integrity but should be validated for that purpose.

KEY QUALITY PARAMETERS FOR BULK FORMULATED POLYSACCHARIDE VACCINE

The bulk formulated polysaccharide vaccine refers to a solution that contains a blend of appropriate amounts of the required monovalent bulk polysaccharides, as well as any buffer salts, excipients, adjuvants, and antimicrobial preservatives present in the vaccine product. Typically, the vaccine is sterilized, usually by filtration, and is ready for filling in the final dosage forms.

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Antimicrobial Preservative

If an antimicrobial preservative is present and is not assayed at a later stage, its concentration can be assayed at this stage using an appropriate validated chemical, physicochemical, chromatographic, or spectroscopic assay. While the specification will depend on the regulatory agency, typically the amount of preservative should not exceed 120% of the expected value, and the manufacturer must demonstrate that the preservative is effective at that concentration (see *Antimicrobial Effectiveness Testing* ⟨51⟩).

Sterility

At this stage, if the expectation is that the material is sterile, then sterility should be demonstrated using an appropriate validated assay such as one of those described in ⟨71⟩. For other manufacturing processes, measurement of some combination of bioburden, endotoxin count, or pyrogenicity may be sufficient.

Polysaccharide Quantity

To ensure correct dilution of the bulk before final filling, and unless other control mechanisms are in place, determination of the content of individual serotypes or total polysaccharide content may be required using appropriate validated physicochemical or immunochemical methods as defined in the bulk polysaccharide section.

pH, Osmolarity/Isotonicity, and Excipients

The final format of a vaccine may be liquid or lyophilized. If the format in this stage does not change during the final fill and is not analyzed later then manufacturers should assay this bulk material for:

- pH (see *pH* ⟨791⟩)
- possibly osmolarity or isotonicity (see *Osmolality and Osmolarity* ⟨785⟩). If approved by a regulatory agency, routine osmolarity testing may be omitted if the manufacturer demonstrated consistency in development and clinical lots.
- excipient content, if present.

KEY QUALITY PARAMETERS FOR POLYSACCHARIDE VACCINES: FINAL FILLS

In accordance with 21 CFR 610.1, "No lot of any licensed product shall be released by the manufacturer prior to the completion of tests for conformity with standards applicable to such product. Each applicable test shall be made on each lot after completion of all processes of manufacture which may affect compliance with the standard to which the test applies. The results of all tests performed shall be considered in determining whether or not the test results meet the test objective, except that a test result may be disregarded when it is established that the test is invalid due to causes unrelated to the product." Test methods should be appropriately verified and validated.

Description and Solubility

Liquid vaccines are typically clear colorless liquids that are essentially free from visible particles. Lyophilized products are typically white or cream-colored powders or pellets that are freely soluble in water and yield clear colorless liquids that are free from visible particles.

Polysaccharide Identity

The manufacturer should demonstrate that all the expected polysaccharides are present in the final fill. The test may be immunochemical, physicochemical, or chemical. Regulation 21 CFR 610.14 requires that the identity test should distinguish the product from other products handled in the same facility. In some cases specific quality attributes of the polysaccharide relating to identity, such as *O*-acetyl content, may also be specified, and should be assayed at this final fill stage if not assessed at an earlier stage. Often polysaccharide identity and quantity can be confirmed and determined by the same assay.

Polysaccharide Quantity

The content of each polysaccharide present in the final lot should be determined by a suitable validated immunochemical or physicochemical method. Typically, Vi and pneumococcal polysaccharide vaccines contain 25 µg of each serotype in a single human dose, whereas meningococcal polysaccharide vaccines contain 50 µg of each serogroup in a single human dose. When immunochemical methods are used, the antisera should be specific for each polysaccharide in the vaccine, including, in the case of the pneumococcal serotypes, immunologically cross-reactive species. Specifications are established on a case-by-case basis, but typically the content of each polysaccharide in the vaccine should be either between 70% and 130% or between 80% and 120% of the label claim.

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Polysaccharide Structural Integrity and Molecular Size

In the absence of suitable validation data showing that no changes occur during filling and storage, the structural integrity and molecular size of the individual polysaccharide components should be assessed as far as is possible following the final fill. Depending on the nature of the polysaccharide and the complexity of the vaccine, the integrity of individual serotypes or serogroups can be established by a combination of immunochemical stability-indicating measurements (e.g., ELISA, rate nephelometry, or physicochemical methods), by size or molecular weight determination alone or in combination with serotype-specific assays, or by quantification of specific groups such as *O*-acetyl groups that have been shown to be critical for immunogenicity. Together with the serotype-specific quantification, these assays act as a surrogate for a potency assay.

pH

The pH of the final fill should be determined for liquid products or for redissolved lyophilized products according to ⟨791⟩ and should meet the requirements of the relevant licensing authority. This attribute should be included in stability-testing programs.

Antimicrobial Preservative

Where applicable, the amount of antimicrobial preservative should be determined by a suitable validated approach. Typically, the value should not exceed 120% of the quantity stated on the label. The approved lower limit should not be lower than the minimum amount shown to be effective throughout the product's shelf life.

Process Impurities

Unless the product has been tested at an earlier manufacturing stage, and depending on the manufacturing process used, process impurities or residuals such as phenol or formaldehyde should be tested by appropriate validated assays. For more information about allowable process impurities, see *Vaccines for Human Use—Bacterial Vaccines* ⟨1238⟩.

Sterility

The sterility of each lot should be ensured according to procedures described in ⟨71⟩ and 21 CFR 610.12. The product should comply with the requirements of the tests.

Pyrogens or Bacterial Endotoxin

Depending on regulatory requirements, the product's endotoxin content or pyrogenicity should be determined and should be shown to meet the relevant specifications. Methods for endotoxin testing are found in ⟨85⟩. Methods for pyrogen testing are found in ⟨151⟩.

Osmolarity and/or Isotonicity, Excipients, and Moisture Content

If the vaccine is a liquid preparation, the pH and osmolarity/isotonicity of each final lot should be tested and shown to be within the pre-approved specifications. For a lyophilized preparation, analysts should measure the pH after reconstitution with the appropriate diluent.

Excipient functional category (sometimes referred to as *functionality*) is a broad, qualitative, and descriptive term for the purpose or role an excipient serves in a formulation. Of greater importance, however, are the quantitative performance requirements (i.e., critical material attributes) of excipients that must be evaluated and controlled to ensure consistent performance throughout the product life cycle. Manufacturers should anticipate lot-to-lot and supplier-to-supplier variability in excipient properties and should have in place appropriate controls if needed to ensure consistent excipient performance (refer to *Excipient Performance* ⟨1059⟩ for guidance).

Regarding moisture content in lyophilized products, as specified in 21 CFR 610.13 each lot of dried product must be tested for residual moisture and must meet and not exceed established limits as specified by an approved method (see also ⟨731⟩ and ⟨921⟩).

Diluent for Lyophilized Products

Data should be provided to support diluent sterility (see ⟨71⟩) and to ensure that adventitious microbial contamination does not occur under the reconstitution conditions (i.e., diluents should not introduce contamination) or during storage conditions as described in the package insert. If an antimicrobial preservative is used (as is the case normally only in multidose products), testing according to ⟨51⟩ is recommended to demonstrate acceptability. Testing may not be required on all lots once process control and consistency have been established.

General Safety

Depending on regulatory requirements, a general safety test may be necessary as set out in 21 CFR 610.11(g), and the product should meet the specifications.

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KEY QUALITY PARAMETERS FOR CARRIER PROTEIN

A number of protein carriers have been used in preclinical and clinical evaluation of conjugate vaccines. Proteins such as diphtheria and tetanus toxoids, which derive from the respective toxins after chemical detoxification with formaldehyde, initially were selected as carriers because of the safety track record accumulated with tetanus and diphtheria vaccination, and these proteins are used today as carriers for meningococcal, Hib, and pneumococcal vaccines in a number of countries worldwide. CRM197, a nontoxic mutant of diphtheria toxin, is also used as a carrier for licensed Hib, pneumococcal, and meningococcal conjugate vaccines and for other vaccines being developed. An outer membrane protein complex (OMPC) of serogroup B meningococcus is the carrier for a licensed Hib conjugate vaccine. An Hib-related protein, Protein D, is the carrier for most of the polysaccharides included in one licensed conjugated pneumococcal vaccine.

Key Quality Parameters for Carrier Protein

Five carrier proteins currently are used for conjugate vaccines approved for use by various regulatory authorities: Diphtheria Toxoid, Tetanus Toxoid, CRM197, *Haemophilus* Protein D, and OMPC. When the carrier protein is a component of an approved vaccine like diphtheria and tetanus toxoids, the first key quality parameters are those defined by the release tests on the concentrated bulks for these components. Other quality parameters include the level of oligomerization (monomer vs. multimeric forms). For carrier proteins that are not licensed as stand-alone vaccines, the list of key quality attributes should at least include identity, sterility or bioburden (depending on the manufacturing process), endotoxins, and purity. In some specific cases, additional quality attributes may require measurement.

CARRIER PROTEIN IDENTITY

The identity of the carrier proteins can be assessed by suitable methods that can be divided into two categories:

- Immunochemical methods: immunoprecipitation (flocculation, radial immunodiffusion, and nephelometry), immunoelectrophoretic methods (rocket immunoelectrophoresis), and immunoenzymatic methods (immunoblots and ELISA)
- Standard physicochemical methods used for other purified proteins: mass spectrometry, peptide mapping, and molecular mass determination.

Using these methods, a sample preparation is compared to a reference preparation to demonstrate consistency. The tests listed here may not be appropriate for toxoid proteins.

STERILITY OR BIOBURDEN

Sterility and bioburden are determined according to procedures described in ⟨71⟩ and ⟨61⟩, respectively, and 21 CFR 610.12.

ENDOTOXINS

To ensure an acceptable level of endotoxin in the final product, manufacturers can determine the endotoxin content of the carrier protein according to ⟨85⟩ and thus can show that endotoxin levels are within acceptable limits. For some products, rabbit pyrogenicity testing (⟨151⟩) may be a more relevant test.

Diphtheria Toxoid

ANTIGENIC PURITY

Depending on the manufacturing process diphtheria toxoid preparation can show different degrees of purity. Typically, antigenic purity for diphtheria toxoid as determined by the flocculation test should be at least 1500 Lf (limit of flocculation) units/mg of protein.

MONOMER, DIMER, OR AGGREGATE CONTENT

Diphtheria toxin is characterized by the presence of dimeric and multimeric aggregation forms that are also present in the corresponding detoxified preparations. Regarding this parameter, analysts can monitor the manufacturing consistency of diphtheria toxoid by determining the content of monomers vs. dimers and other aggregates using a suitable method such as SEC-HPLC coupled with a static light scattering detector. In some cases, because of the low purity of the preparation the HPLC profile may result in a broad peak that cannot be resolved into the contribution of the individual species.

Tetanus Toxoid

ANTIGENIC PURITY

Typically, the antigenic purity of tetanus toxoid as determined by the flocculation test should be at least 1500 Lf units/mg of protein.

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MONOMER, DIMER, OR AGGREGATE CONTENT

The detoxification process for tetanus toxin results in oligomerization to an extent that depends on the process conditions. Similar to diphtheria toxoid, the physicochemical consistency of tetanus toxoid can be monitored by the determination of monomeric vs. dimeric forms and other aggregates using suitable methods like SEC-HPLC coupled with static light scattering detection. Other methods like ultracentrifuge analysis can be applied but may be less suitable for routine testing.

CRM197

CRM197 protein is a nontoxigenic diphtheria toxin isolated from the supernatant of cultures of *Corynebacterium diphtheriae* C7(β197)$^{tox-}$ and purified by a sequence of chromatographic and diafiltration steps. A guideline for production and control of bacterial proteins used in vaccine manufacturing is given in ⟨1238⟩. Recombinant CRM197 should meet requirements for nonrecombinant material, although additional characterization appropriate for recombinant proteins may be required.

PURITY

The purity of CRM197 batches should be determined via suitable methods, e.g., HPLC (see ⟨621⟩), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩), or *Capillary Electrophoresis* ⟨1053⟩. Typical expectations are that the purity of CRM197 should be at least 90% and often greater than 95%.

DEGREE OF NICKING

CRM197 contains an exposed loop of three arginine residues that is clipped by proteases present in the culture medium, resulting in a so-called nicked form. The manufacturing process should demonstrably be able to regularly produce CRM197 with a consistently low degree of nicking. In the presence of a reducing agent like dithiothreitol, the nicked form breaks down into two distinct polypeptides called fragments A and B that can be easily detected by SDS-PAGE, which accordingly is a suitable method to determine the degree of nicking. In a validated process testing may be needed as an in-process control.

Haemophilus Protein D

Haemophilus Protein D is obtained as a recombinant protein from *E. coli* fermentation that, after extraction from the cells, is purified by a series of chromatographic and diafiltration steps and finally is sterile filtered. Routine release tests for protein D include identity, purity, sterility, protein content, and endotoxin content. Host cell proteins and host cell DNA should also be tested unless process validation has shown consistent clearance.

PURITY

Purity should be monitored with an appropriate test such as HPLC, SDS-PAGE, or capillary electrophoresis (CE).

Outer Membrane Protein Complex

The OMPC of *Neisseria meningitidis* group B is derived from meningococcal serogroup B bacterial cells by extraction with buffer-containing detergent. Analysts can monitor manufacturing consistency by determining the OMPC composition with SDS-PAGE or another suitable method.

ENDOTOXIN CONTENT OR PYROGENICITY

To ensure an acceptable level of endotoxin in the final product, analysts can determine the endotoxin content of the carrier protein and can show that it falls within acceptable limits according to ⟨85⟩. Alternatively, OMPC preparations should pass the rabbit pyrogenicity test following injection into rabbits at, typically, 0.25 µg/kg of body mass (see ⟨151⟩).

MENINGOCOCCAL OMPC REQUIREMENTS—PURITY AND LIPOPOLYSACCHARIDE CONTENT

The composition of meningococcal OMPC carrier should be monitored for consistency by SDS-PAGE or by another suitable method. Typically, the lipopolysaccharide (LPS) content should not exceed 8% by weight. Suitable methods for LPS determination include HPLC, colorimetric analyses, SDS-PAGE, and GC-MS.

KEY QUALITY PARAMETERS FOR ACTIVATED INTERMEDIATES

Different chemical strategies can be applied to the synthesis of glycoconjugate vaccines. Two main approaches have traditionally been employed for glycoconjugate vaccines preparation: one is based on random chemical activation along the chain of the native or slightly size-reduced polysaccharide, followed by conjugation; the other is based on selective activation of end groups of oligosaccharides generated by controlled fragmentation of the native polysaccharide and subsequently coupling to the protein carrier. Depending on the conjugation chemistry, a chemical spacer can be used to facilitate the coupling of the protein to the saccharide antigens, and, in some cases, prior derivatization of the protein carriers is also required. In some

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cases the activated or derivatized polysaccharide or oligosaccharide is isolated and represents an intermediate of the glycoconjugate vaccine manufacturing process. In order to ensure a reproducible product by means of consistent application of the appropriate conjugation stoichiometry, manufacturers should use appropriate methods to determine the degree of activation or derivatization of the poly- or oligosaccharide and of the carrier protein.

Degree of Activation of Activated Polysaccharide

If appropriate, different activation or derivatization strategies can be applied to poly- or oligosaccharides in order to make them suitable for covalent coupling to the carrier protein. In some cases the hydroxyl groups of the polysaccharide first are reacted with cyanogen bromide or CDAP or carbonyldiimidazole to form active esters. These active intermediates then can be reacted *in situ* with adipic acid dihydrazide or other bifunctional amines to introduce an amino linker. Some bacterial polysaccharides possess carboxyl or phosphate groups that might be used for introduction of an amino linker using a carbodiimide-mediated chemistry. Subsequent coupling to the carboxyl groups of the carrier protein to obtain the desired glycoconjugate can be performed using, for example, a soluble carbodiimide-mediated chemistry. Alternatively, the amino linker incorporated into the polysaccharide structure can be further derivatized to obtain a bromo-acyl or a maleimido function that is amenable to coupling with a thiol group that is present or previously was incorporated into the carrier protein.

In some other cases aldehyde groups can be introduced into the polysaccharide structure by reaction of vicinal hydroxyl groups with the oxidizing reagent sodium metaperiodate ($NaIO_4$). Depending on the polysaccharide structure, the $NaIO_4$ treatment can also be used for simultaneous controlled depolymerization and aldehyde group generation. The poly- or oligosaccharides-containing aldehyde groups then can be covalently coupled to lysine residues and the *N*-terminal amino groups of the carrier protein by reductive amination in the presence of sodium cyanoborohydride ($NaBH_3CN$) or other reducing agents that are selective for Schiff bases.

Some manufacturing strategies are based on the controlled hydrolysis of the native polysaccharides to produce oligosaccharides that can be specifically derivatized by a sequence of steps that lead to the introduction of an active ester function at their reducing ends. The desired conjugate is then obtained by reaction of the activated oligosaccharides with the lysine residues and *N*-terminal amino groups of the carrier protein.

To ensure a consistent conjugation stoichiometry, and therefore a consistent manufacturing process, manufacturers should determine the level of derivatization or activation of the poly- or oligosaccharide intermediates. Appropriate methods for determination of the newly introduced chemical functions into the saccharide structures should be in place and could include, for example, colorimetric or other suitable methods. In cases where the activated polysaccharide is conjugated without isolation, consistency in the degree of polysaccharide activation may also be demonstrated as part of process validation or reflected by characteristics of the final conjugate bulk.

The calculation of the degree of poly- or oligosaccharide derivatization may require determination of the total saccharide quantity that can be achieved by applying, for example, HPAEC-PAD, colorimetric assays, or other suitable methods. In a validated process where production consistency has been established, and depending on the conjugation chemistry used and the results of clinical trials, testing may be used as an in-process control. Residual unconjugated linker that could interfere with subsequent steps should be controlled via measurement or process validation.

Molecular Sizing of Activated, Derivatized, or Processed Polysaccharide

The molecular size and degree of polymerization of the poly- or oligosaccharide intermediates depends on the particular manufacturing process and should be measured because these attributes can affect the consistency of the conjugation process. Suitable tests should be applied to intermediate pools that are selected on the basis of the different manufacturing processes. Examples of suitable methods for profiling molecular sizes and determining the degree of polymerization of poly- and oligosaccharides are: SEC coupled with UV, RI, or static light scattering detectors; colorimetric assays based on total and end group determination; HPAEC-PAD; or NMR spectroscopy. In a validated process, testing can be used as an in-process control.

Degree of Activation of Activated Carrier Protein

As mentioned above, some manufacturing procedures for glycoconjugate vaccines may also require activation of the protein carrier. This process step introduces into the protein side chains additional functional groups that react with the poly- or oligosaccharide intermediates activated with the proper functional group. In general, such functional groups are introduced by derivatization of protein amino acid side chains like glutamic or aspartic acid with a bifunctional reagent (e.g., adipic acid dihydrazide or hydrazine) so that a highly nucleophilic hydrazide group becomes available for coupling with the polysaccharide. In other manufacturing strategies, the lysine side chains of the carrier protein can be derivatized to introduce different reactive groups (e.g., bromo-acyl, thiol, or maleimido groups). Appropriate methods for determination of the newly formed chemical functions introduced into the carrier proteins should be in place and may include spectrophotometric assays and mass spectrometry. The calculation of the degree of activation or derivatization of the carrier protein may also require the determination of the total protein quantity (e.g., by colorimetric assays or other appropriate methods). In a validated process where production consistency has been established, and depending on the conjugation chemistry used and the results of clinical trials, testing may be used as an in-process control. Depending on the conjugation chemistry used (i.e., immediate conjugation after activation), consistency in degree of carrier protein activation may also be demonstrated as part of process validation or reflected by characteristics of the final conjugate bulk.

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Carrier Protein Monomer Content

In some cases the procedures for protein carrier activation or derivatization may result in a certain degree of covalent aggregation of the carrier itself, and this should be monitored with appropriate tests like SEC-HPLC coupled with static light scattering detection, SDS-PAGE, matrix-assisted laser desorption–ionization mass spectrometry, or other suitable tests.

KEY QUALITY PARAMETERS FOR MONOVALENT BULK CONJUGATE (DRUG SUBSTANCE)

Conjugation

Conjugation of the polysaccharide antigen to the carrier protein or protein complex is the critical component of the manufacturing process for conjugate vaccines. A general overview of a conjugation process is presented in ⟨1238⟩. In addition to developing a description of critical processing equipment, reagents, and processing steps, manufacturers should provide the rationale for the conjugation chemistry selected and the purification steps, if any, used to remove unwanted reaction components. Clearance of product-related impurities (e.g., unconjugated polysaccharide or unconjugated carrier protein) should be monitored and controlled.

Depending on the nature of the manufacturing process, the monovalent conjugate bulks can be considered to be the drug substance or can be considered a process intermediate.

During conjugation, the reactive functional groups present on the polysaccharide antigen are reacted with the functional groups located on the carrier protein or carrier complex to form stable, covalent bonds. Many different types of chemistries are used, including reductive amination, thio-alkylation, or CDAP chemistry. The choice of chemical method should be based on the availability of functional groups, either naturally occurring or introduced via an activation or side chain loading process, and the ability to control the manufacturing process to produce a consistent and stable product.

Although the conjugation process may be conceptually straightforward, the process should be well controlled. The process typically consists of mixing the activated polysaccharide with the selected carrier and allowing the components to react. Depending on the nature of the chemical reaction, an additional chemical reactant may be needed to complete the reaction or stabilize the conjugate product. For example, in the case of reductive amination, it may be necessary to add a reducing agent to convert the linkage from a relatively unstable Schiff base to a more stable secondary amine. Chemical deactivation or capping of residual reactive groups may also be required. Finally, residual levels of unreacted components such as free protein, free polysaccharide, chemical reagents, and byproducts should be removed from the process via validation or should be monitored by testing. Regulatory authorities may request stability evaluation of these intermediates because the data may support stability predictions for multivalent vaccines for which data collection is more difficult.

To define and control the conjugation process, the manufacturer should establish targets for process parameters and tolerances for all critical process steps where possible, including extent of activation, charge ratios for each reaction component, reaction time, reaction temperature, reaction pH, and mixing conditions. Additionally, tolerances for the purity of each of the reaction components should be established, including the polysaccharide, carrier protein, and any chemical components as noted above.

The common key quality attributes for monovalent conjugates, the rationale for monitoring these parameters, and suitable test methods are described below.

Polysaccharide Identity

Polysaccharide identity confirms that the correct antigen was used during the manufacturing process and that no critical epitope was lost during conjugation. Polysaccharide identity should be confirmed using a suitable immunological or chemical method. Examples of immunological methods include ELISA, immunoblot analysis, and rate nephelometry. The specificity of the test method must be ensured by selection of appropriate reagents. Alternatively, the identity of the polysaccharide can be confirmed using a chemical or physical method such as HPLC, HPAEC-PAD, GC, or NMR if acceptable specificity can be demonstrated and it can be shown that the carrier protein does not substantially interfere with the identification of the polysaccharide.

Carrier Protein Identity

Depending on the nature of the manufacturing process and the manufacturing controls, it may be necessary to confirm the identity of the carrier protein, e.g., during a manufacturing process for a multivalent product in which different antigens are conjugated to different carrier proteins within the same facility. Carrier protein identification can also be performed using an immunological method such as ELISA or, if possible, an appropriate chemical method such as peptide mapping (see *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩). If appropriate, the carrier identity can be evaluated in the same assay that is used for the identity of polysaccharide.

Polysaccharide Quantity

Polysaccharide quantity or concentration must be confirmed for all lots of monovalent conjugate because it is directly related to the product dose. Polysaccharide yield can also be a useful marker for process consistency. A variety of methods are available and suitable for use in determining the polysaccharide concentration. These include colorimetric methods such as the phenol–sulfuric acid, orcinol, and anthrone–sulfuric acid assays and monosaccharide analysis following hydrolysis by HPAEC-PAD, HPLC with fluorescence detection (HPLC-FD), or GC. Immunological methods that may be suitable include ELISA or rate nephelometry. The suitability of these methods depends on the availability of appropriate reagents. The choice of method

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should be made on the basis of precision and accuracy. Interference from the carrier protein must be avoided. Additionally, the chemistry of the polysaccharide antigen should be considered when analysts select the method. For example, the phenol–sulfuric acid assay may not be suitable for use if the antigen is composed largely of amino sugars or sialic acid. Suitable methods for polysaccharide quantification are listed in previous sections of this chapter.

Carrier Protein Quantity

The concentration of the carrier protein must be confirmed for all lots of monovalent conjugate. Conjugate vaccines are typically formulated based on the polysaccharide concentration, not the carrier protein concentration. However, the concentration of the carrier protein is needed to determine the polysaccharide–protein ratio, a key indicator of process consistency.

Analysts should select a test method that is specific for the carrier protein and does not suffer from interference from the polysaccharide components. Suitable methods may include amino acid analysis (see *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩), colorimetric protein tests such as the bicinchoninic acid assay or UV absorbance (see ⟨1057⟩ for both types of methods), or the protein-specific output from HPSEC with static light scattering, RI, or UV detection.

Polysaccharide–Protein Ratio

As noted in *Carrier Protein Quantity,* the polysaccharide–protein ratio may be an indicator of process consistency. Therefore, tolerances should be established for two of the three parameters: polysaccharide concentration, protein concentration, and polysaccharide–protein ratio. It is not necessary to establish limits for all three parameters because the polysaccharide–protein ratio is typically calculated from the measured polysaccharide and carrier protein concentrations. Because the downstream processing and dilutions usually are based on the amount of polysaccharide present in the monovalent bulks, the limit for the polysaccharide content can be based on the minimum concentration required for downstream processing.

Molecular Size Distribution or Integrity or Proof of Covalency

The molecular size of the conjugate is a key indicator of process consistency. Unusually small conjugates may indicate incomplete conjugation whereas unusually large conjugates may indicate aggregation and may result in a loss of yield during downstream filtration steps. The average molecular size and the size distribution should be measured using appropriate sizing methods such as SEC, HPSEC, HPSEC with static light scattering or RI detection, or analytical ultracentrifugation. The choice of the method should be made on the basis of the expected size of the conjugate and the availability of a chromophore for detection.

Proportion of Free (or Unconjugated) Polysaccharide

The proportion of unconjugated polysaccharide must be monitored for each lot of monovalent conjugate because of the possibility that the presence of a large amount of unconjugated polysaccharide may suppress the immune response to the antigen. Additionally, the presence of free polysaccharide is a key indicator of process consistency and is an indirect measure of covalent attachment to the carrier. Measurement of the proportion of unconjugated polysaccharide can be used as a stability-indicating test if appropriately validated.

To measure the level of unconjugated polysaccharide, analysts must separate the unconjugated polysaccharide from the conjugated polysaccharide. This can be achieved chromatographically, or by chemical precipitation of the conjugate with acid or detergents, aluminum adsorption, capillary electrophoresis, gel filtration, centrifugal ultrafiltration, solid-phase extraction, or immunoprecipitation. The amount of free polysaccharide then can be quantitated using the method that was used to quantitate the total polysaccharide level, if that method is sufficiently sensitive, by UV detection if a chromophore is present, or by immunological or appropriate physicochemical methods.

The level of unconjugated polysaccharide must be measured at release and during stability testing because deconjugation is a potential degradation mechanism.

Proportion of Unconjugated Carrier Protein

The level of unconjugated carrier protein must be monitored for each lot of monovalent conjugate because this level is a key marker of process consistency and is an indirect measure of covalency. To measure the amount of unconjugated protein, the unconjugated protein must be separated from the conjugate. This can be done chromatographically or by electrophoresis (slab or capillary). Once it is separated, the amount of unconjugated protein can be monitored by UV or by a colorimetric method (see ⟨1057⟩). Method selection should be based on sensitivity, precision, and specificity for unconjugated protein.

Unreacted Functional Groups

During the conjugation process, reactive functional groups on the polysaccharide react with functional groups on the carrier protein. However, the reaction is typically not driven to completion, and a process of capping of remaining reactive groups may be required, depending on the nature of the residual reactive groups, the conjugation chemistry used, and manufacturing process optimization. The method chosen to cap reactive groups depends on the conjugation chemistry employed. Even so, some reactive groups may still remain on the conjugate even after the reaction is quenched by reduction, or after remaining reactive groups have been chemically capped. Safety concerns, if any, depend on the nature of the reactive groups and the level of reactive groups that remain. The level of residual reactive groups should be monitored as a measure of process

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consistency unless process validation has shown that unreacted functional groups detectable at this stage are removed during subsequent manufacturing processes. Additionally, the presence of residual reactive groups may affect product stability during storage.

The test method used to evaluate residual reactive groups depends on the activation chemistry that is used and the nature of the polysaccharide antigen. Appropriate methods may include gas chromatography, HPLC with fluorescence, or UV detection following hydrolysis.

Residual Reagents

Consistency in the amount of residual reagents from the conjugation chemistry can be demonstrated during process development, and the process can be validated for their clearance. This validation includes not only unconjugated polysaccharide and protein but also buffers, salts, small-molecule reaction components, and byproducts generated during conjugation. Provided that consistent levels of residual solvents are recovered, such testing may serve as an in-process control.

Sterility or Bioburden

Depending on the manufacturing process, the monovalent conjugate bulks should be tested for bioburden or sterility. If the monovalent conjugate bulks are subjected to additional process steps with no process holds, in some cases it may be appropriate to perform the sterility or bioburden test at a downstream step.

Bacterial Endotoxins

The monovalent conjugate bulks must be tested for bacterial endotoxins. If the monovalent conjugate bulks are subjected to additional process steps with no process holds, in some cases it may be appropriate to perform the endotoxin test at a downstream step.

FORMULATED AND ADJUVANTED (IF APPROPRIATE) CONJUGATE BULKS

Monovalent conjugate bulks can be individually adsorbed and formulated as monovalent bulks before mixing during preparation of the final vaccine.

Adjuvant Content

If an adjuvant has been added to the conjugate bulk, its content should be determined by an appropriate method. If aluminum is used as an adjuvant, typical maximum values are 0.85 mg of aluminum per dose, although higher limits up to 1.25 mg of aluminum per dose may be accepted if justified, and lower limits may apply according to governing agency requirements.

Polysaccharide Content

Assessment of polysaccharide content can be required but is often difficult at this stage because of the presence of adjuvants or excipients. Several methods are available and are suitable for use in determining the polysaccharide concentration. These include colorimetric methods such as the phenol–sulfuric acid, orcinol, and anthrone–sulfuric acid assays; and post-hydrolysis monosaccharide analysis by HPAEC-PAD, HPLC-FD, or GC. Suitable immunological methods include ELISA or rate nephelometry. If possible, the same method must be used for both this step and the conjugate bulk step. The choice of method should be based on precision, accuracy, and specificity. Interference from the matrix must be avoided. Additionally, the chemistry of the polysaccharide antigen should also be considered when analysts select the method.

Free (or Unconjugated) Polysaccharide

Free polysaccharide content must be measured as a release test for monovalent formulated bulk if the free polysaccharide cannot be accurately or precisely measured in the final product. For most applications, polysaccharide testing is a marker of consistency; i.e., it is monitored for each bulk in order to establish production consistency. The test may be omitted when manufacturing consistency has been demonstrated, or the test can be used as an in-process control. If free polysaccharide could be adsorbed on adjuvant, a desorption step using, for example, phosphate buffer, may be required. The unconjugated polysaccharide must be separated from adsorbed and nonadsorbed conjugated polysaccharide. Adsorbed conjugate can be removed by centrifugation, and nonadsorbed conjugate can be eliminated chromatographically; by chemical precipitation of the conjugate with acid or detergent precipitation or aluminum adsorption; by immunochemical precipitation with anti–carrier antibodies; by capillary electrophoresis; or by gel or membrane filtration or ultrafiltration. The amount of free polysaccharide then can be quantitated using the same method that was used to quantitate the total polysaccharide level if the latter is sufficiently sensitive and accurate. Quantitative tests for polysaccharide include colorimetric methods such as the phenol–sulfuric acid, orcinol, and anthrone–sulfuric acid assays; and monosaccharide analysis following hydrolysis by HPAEC-PAD, HPLC-FD, or GC. Suitable immunological methods include ELISA or rate nephelometry.

Results should be expressed as the percentage of unconjugated polysaccharide vs. the total content experimentally determined, or, if this value cannot be determined experimentally, it may be possible to calculate it from a theoretical value.

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The level of unconjugated polysaccharide must be measured during stability studies because deconjugation is a potential degradation mechanism.

Level of Adsorption to Adjuvant

The level of adsorption in monovalent formulated bulks must be performed as a release test if it cannot be performed on the final product. If it is not a release test then the adsorption test is a marker of consistency, and the test is performed on each bulk in order to establish production consistency. After consistent production has been demonstrated, the test can be omitted.

After product centrifugation, nonadsorbed conjugate is quantified in the supernatant. The adsorbed conjugate then is quantified by a suitable validated method, which can be the same test used for the total conjugate content. If the physical or chemical method used for the quantification is not specific for the conjugated form of the saccharide, the amount of free polysaccharide is subtracted from the total polysaccharide in order to determine the quantity of adsorbed conjugate. Otherwise an immunologically specific method can be used (e.g., ELISA). Results can be expressed as the percentage of nonadsorbed conjugate vs. the total content experimentally determined. If it is not possible to determine the amount of nonadsorbed conjugate experimentally then it may be possible to calculate it from a theoretical value. The level of adsorption to adjuvant must be measured during stability testing.

Sterility

The sterility of each lot should be measured according to procedures described in ⟨71⟩ and 21 CFR 610.12.

KEY QUALITY PARAMETERS FOR CONJUGATE VACCINE DRUG PRODUCT

Description and Solubility

Each container in each final fill or drug product should be inspected visually (manually or with automatic inspection systems), and containers that show abnormalities such as improper sealing, lack of integrity, or turbidity should be discarded. Similarly, the presence of clumping or particles may indicate a product failure.

Polysaccharide Identity

Polysaccharide identity tests confirm that the correct antigen was used during the manufacturing process. Polysaccharide identity should be confirmed using a suitable immunological or physicochemical method, e.g., ELISA, immunoblots, or rate nephelometry. The specificity of the test method must be ensured by use of appropriate reagents. Acceptable specificity must be demonstrated, and tests must show that the carrier protein does not substantially interfere with the identification of the polysaccharide. Assays based on hydrolysis and chromatographic identification of saccharide components (e.g., HPAEC) after polysaccharide hydrolysis may be acceptable.

Polysaccharide Quantity

Typically, monovalent conjugate vaccines contain 10 µg of saccharide, and multivalent vaccines contain between 1 and 10 µg of each serotype or serogroup per single human dose. Assessment of the content of polysaccharide may be difficult because of the presence of adjuvant or excipients, especially when multiple components are present. The amount of each polysaccharide may be required in order to calculate the free polysaccharide content and the proportion of unadsorbed conjugate, or this information may be used during processing of the vaccine's final formulation. Polysaccharide quantity or concentration must be confirmed for all types of monovalent conjugates because it is directly related to the product dose. A variety of methods are available and suitable for use in determining the polysaccharide concentration [see discussion of polysaccharide quantitation in the *Key Quality Parameters for Monovalent Bulk Conjugate (Drug Substance)* section above].

Carrier Protein Identity (if Appropriate)

Depending on the nature of the manufacturing process and the manufacturing controls, if the identity of the carrier protein has not been confirmed at an earlier stage, it may be necessary to do so before product release. For example, an identity test for the carrier protein could be necessary during the manufacturing process for a multivalent product for which different antigens are conjugated to different carrier proteins within the same facility. Carrier protein can be identified using an immunological method such as an immunoblot or ELISA, or using an appropriate chemical method such as peptide mapping. If appropriate, the carrier's identity can be evaluated in the same assay used to identify the polysaccharide.

Molecular Size (if Feasible or Appropriate)

If the molecular size distribution has not been established for the individual monovalent bulk conjugates used in the drug product formulation, the molecular size distribution of the conjugates must be determined in the final fill or drug product. The molecular size of the conjugate is a key indicator of process consistency. The average molecular size and the size distribution should be measured using appropriate sizing methods such as SEC, HPSEC, HPSEC with static light scattering or RI detection, analytical ultracentrifugation, or dynamic light scattering. The choice of the method should be based on the expected size of the conjugate and the availability of a chromophore or fluorophore for detection. In the case of molecular size distribution

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determinations at the final fill or in drug products composed of multivalent polysaccharides, serotype-specific detection methods may be required for the individual monovalent conjugates. Molecular size is a sensitive indicator of conjugate stability, and, where possible, it should be measured during stability studies.

Proportion of Free (or Unconjugated) Polysaccharide

To measure the level of unconjugated polysaccharide, analysts must separate the unconjugated polysaccharide from the adsorbed and nonadsorbed conjugated polysaccharide and interfering substances. If unconjugated polysaccharide may be adsorbed to adjuvant, prior desorption with, for example, phosphate buffer is required. Methods for separation and measurement of conjugated and unconjugated polysaccharide are described above in *Formulated and Adjuvanted Conjugate Bulks*. The level of unconjugated polysaccharide is a stability-indicating measurement because deconjugation is a potential degradation mechanism and should be measured during stability studies. However, assessing the level of unconjugated polysaccharide and the stability of complex multivalent products is technically demanding, and alternative approaches to assessing antigen integrity include molecular size, *O*-acetyl content, or immunological measurement. Specific assays may provide partial but overlapping information and should be matched to the product. Free saccharide data obtained for individual monovalent conjugates may also prove valuable and predictive.

pH, Osmolarity/Isotonicity, and Excipients

If the vaccine is a liquid preparation, the pH and osmolarity/isotonicity of each final lot should be tested and shown to be within the pre-approved specifications. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent. Residual moisture in lyophilized products should be determined. See comments above regarding excipient functionality.

Adjuvant Quantities (if Appropriate)

If an adjuvant has been added to the conjugate bulk, its content should be determined by an appropriate method. If aluminum compounds (such as aluminum hydroxide or hydrated aluminum phosphate) are used as adjuvants, the amount of aluminum should not exceed 1.25 mg per single human dose or as otherwise required by the governing agency (see also 21 CFR 610.15).

Antimicrobial Preservative (if Appropriate)

During product development manufacturers should consider the stability of the chosen preservative and possible interactions between the vaccine components and the preservative. If a preservative has been added to the vaccine, the content of preservative should be determined by an appropriate method (see *Antimicrobial Agents—Content* ⟨341⟩). The amount of preservative in the vaccine dose should be shown neither to have any deleterious effect on the antigen nor to impair the safety of the product in humans. If present, the amount must be NLT the minimum amount shown to be effective and typically should be NMT 120% of the amount stated on the label.

Moisture Content (Lyophilized Products)

If the vaccine is freeze-dried, the average moisture content should be determined by an appropriate method. Values should be within limits established during the product's stability studies. Typically, the average residual moisture content should be NMT 2.5%, and no vial should be found to have a residual moisture content of 3% or greater.

Sterility

The sterility of each lot should be determined according to procedures described in ⟨71⟩ and 21 CFR 610.12.

Pyrogens and Endotoxins

Endotoxin (see ⟨85⟩) content or pyrogenic activity (see ⟨151⟩) should be within approved product specifications.

Diluent for Reconstitution of Lyophilized Vaccines

Manufacturers should generate data that show that adventitious microbial contamination does not grow under the reconstitution conditions (e.g., with diluents that will be used for reconstitution) or under specified storage conditions. A preservative is not normally required for single-dose vials when the product will be used soon after reconstitution, but multidose vials do require preservatives. Testing in alignment with ⟨51⟩ is recommended to demonstrate acceptability, but testing may not be required routinely after process control and consistency have been established.

General Safety or Abnormal Toxicity

The general safety or abnormal toxicity for vaccines should be established by appropriate evaluation and should be consistent with levels found to be acceptable in vaccine lots that were used in clinical trials.

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⟨1235⟩ VACCINES FOR HUMAN USE—GENERAL CONSIDERATIONS

INTRODUCTION

Vaccines have been used for centuries to immunize individuals against pathogenic organisms with the goal of preventing the associated disease. Vaccines are biological products that contain antigens capable of inducing a specific and active acquired immune response in the body. Antigens present in vaccines are processed by specialized cells in the body's immune system, resulting in the development of blood proteins known as antibodies (i.e., humoral immunity) or specialized lymphocytes (i.e., cell-mediated immunity) or both. Therefore immune responses may be antibody mediated, cell mediated, or both. Thus, antigens are critical for vaccine function and generally consist of a portion of the pathogenic organism, or an attenuated form of the whole microorganism. In the case of DNA-based vaccines (currently under development), the vaccine would contain nucleotide sequences (genetic material) that encode microbial antigens.

Examples of types of licensed vaccines appear in *Appendix 1*. A current list of vaccines licensed in the United States is posted at www.fda.gov/cber/.

Vaccines can be of various types, depending on their design and processes involved in their manufacture. Vaccines for human use may contain whole killed or attenuated organisms (e.g., bacteria or viruses) or contain antigens derived from portions of a pathogen, either by partitioning and purification or derived using recombinant technology (*Table 1*). Some polysaccharide vaccines are conjugated to a carrier in order to enhance their immune response.

Table 1. Bacterial and Viral Vaccines

Live attenuated whole cell or virus[a]
Inactivated/killed[b]
Whole cell or virus[c]
Recombinant proteins[d]
Subunit[e]
Polysaccharides
Proteins
Modified toxins

[a] Live attenuated bacterial or viral vaccines are weakened (attenuated) forms of a pathogen. They contain antigens that are similar to disease-causing microbes. They may be derived from the pathogen itself, or from a different organism that contains antigens that cross-react with the virulent microbe (e.g., vaccinia and variola).
[b] Inactivated bacterial and viral vaccines are produced by growing cells of disease-causing bacteria or viruses in cell substrates and subsequently inactivating them to prevent replication in the recipient.
[c] Inactivated/killed whole-cell or virus vaccines consist of the entire microorganisms after they have been inactivated. These preparations may or may not be partially or completely purified.
[d] Recombinant protein viral and bacterial vaccines are derived from host cells that have been transformed with expression vectors that carry genes that encode antigenic material from infectious agents. The expression cells are grown in bioreactors to produce the recombinant antigenic material.
[e] Subunit vaccines are extracts from inactivated/killed viruses or bacteria. Subunit-type vaccines generally undergo some degree of purification.

In addition to antigen(s), vaccines may contain several other components, such as adjuvants that enhance the immune response to the vaccine antigen, preservatives to prevent bacterial or fungal contamination of multiple-dose vials, or other excipients needed for pharmaceutical manufacturing or vaccine stabilization. Residual components from the manufacturing process also may be present in vaccine preparations. Examples of these categories are listed in *Table 2*.

Table 2. Vaccine Components

Antigens
Whole organisms
Components/subunits
Recombinant proteins
Adjuvants
Aluminum salts
Antimicrobial preservatives
Thimerosal
2-Phenoxyethanol
Benzethonium chloride
Phenol
Stabilizers
Salts

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Table 2. Vaccine Components *(continued)*

Amino acids
Sugars
Proteins
Other
Manufacturing residuals
Cell-derived residuals
Materials of animal origin
Antibiotic residuals
Inactivating chemical agents
Other

Different vaccine antigens are often combined in one final formulation in order to elicit immunity against multiple diseases and to reduce the number of separate administrations needed to achieve immunity to the various vaccine antigens.

Despite the multiple forms vaccines may take, several common features characterize the manufacture and testing of vaccines. This chapter focuses on commonalities throughout the manufacturing process, from raw material qualifications to final release tests.

Regulations and Standards

Vaccines are regulated by FDA as biological products. The general requirements are listed in national laws and international guidances. For the U.S., national requirements are codified in 21 CFR, the 200 and 600 sections, with additional recommendations available in FDA *Points to Consider* and *Guidance* documents (www.fda.gov). International guidances are available from the International Conference on Harmonization (ICH) (www.ich.org; see *Appendix 2*) and the World Health Organization. New methodologies are continually being developed and validated and will be included in *USP* as they become available. Reference standards are available from USP and FDA.

OVERALL MANUFACTURING PLAN

When considering the overall plan for manufacturing a vaccine, manufacturers need to consider the following factors:

- Physical facilities;
- Raw materials and process aids;
- Actual manufacturing process, including
    1. initial process (production of virus/bacteria and recombinant materials);
    2. downstream processes (purification or chemical modification, if applicable);
- Antigen modifications such as conjugation or toxoiding;
- Storage of process intermediates and final bulk;
- In-process and final product testing regimens and control schemes;
- Addition of adjuvants, if applicable;
- Formulation and filling;
- Container–closure system; and
- Stability program that supports the dating period of the product.

Quality systems are needed to support the following manufacturing process development: specifications for raw materials, process intermediates, and final product; change control; and failure investigations and complaints. All of these elements are important in the life cycle of the vaccine product.

The overall goal of a comprehensive manufacturing program is to consistently produce a vaccine that is safe and effective. Concurrently with clinical development of the vaccine, the manufacturing process is refined and the process and testing methods are validated for consistency. This includes systems to control changes to the process or inputs. Manufacturers should expect that changes will be required during the vaccine's manufacturing life cycle, and manufacturers necessarily will use data from development and routine manufacturing to assess the process as well as proposed changes. The manufacturers should adopt systems that continually evaluate all aspects of manufacturing to identify unanticipated changes in vaccine quality and to assess them as quickly as possible.

Manufacturing Facilities and Systems

Manufacturers should have a general layout of manufacturing facilities, including diagrams that show the following: flow of raw materials and process inputs; movement of product, intermediates, waste streams, and personnel; and air flows and pressurization levels. These diagrams assist in minimizing the risk of potential product contamination from various sources. These sources can include cross-contamination from other products, contamination from different batches of the same product, and extraneous contamination from microorganisms and personnel. Evaluation of the flow diagrams can assist with strategies for development of engineering controls, personnel procedures, and monitoring systems to enable compliance with Good

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Manufacturing Practices (GMPs). Analysis of potential risks may also provide insights about what information should be recorded in batch documentation to facilitate consistent manufacture and also to facilitate failure investigations. Together, physical facilities, procedures, personnel, training, and quality systems make up the GMP environment in which a vaccine will be produced.

Manufacturing Process

The manufacturing process includes process inputs such as raw materials and processing aids and unit operations comprising both the initial and downstream processing steps. A process flow map for the manufacturing process is useful and assists in validation of the manufacturing process. This map shows all unit operations, the inputs to each operation, and the outputs to subsequent manufacturing steps. Analytical testing done at relevant steps and the specifications required to proceed to the next stage of processing may be added to the map. A process map also supports a processing space to facilitate a rugged process, i.e., one based on suitable characterization studies to establish boundaries within which manufacturing can occur to promote unchanged safety and efficacy outcomes.

The process flow map should include all steps from making the seed/cell bank (described below) to formulation and filling of the final product. The validation strategy should include the steps that require validation, along with identification of the process space, associated critical process parameters (CPP), and critical quality attributes (CQA). The critical process parameters are those that directly affect core quality attributes needed to successfully manufacture a batch of product. Some manufacturers identify other processing parameters that are important for processing but do not affect critical quality attributes. These important but noncritical factors help identify the process development space, can contribute to the development of a rugged process, or can be useful when the company assesses processing deviations. The concepts of quality by design and exploration of the process space are relatively new to the biologics/vaccine industry but are becoming considerations for the overall development-planning process.

Manufacturing Surveillance

Manufacturing surveillance is the continual observation of how the process and the resulting product are performing. This section is not exhaustive; rather, the points raised here outline the types of considerations recommended for a manufacturer during development of a vaccine. Manufacturing surveillance includes the following:

- Periodic review of the performance of the manufacturing process;
- Analytical assays;
- Stability programs;
- Product complaints;
- Adverse event reports;
- Product failure investigations;
- Atypical or deviation events.

Taken as a whole, these activities allow a manufacturer to assess the state of the process and product and to evaluate which, if any, operations need to be modified. These same systems also provide a surveillance matrix to evaluate changes. In any of these programs it is also valuable to develop additional characterization assays that are not used for process intermediate or product release purposes but may be used for further evaluation when additional information is needed or desired. These additional assays for characterization are often based on different underlying analytical procedures to provide different ways to evaluate materials.

Routine surveillance processes are increasingly implemented to attempt to detect changes in processes before any critical quality attributes are adversely affected. Not all vaccine processes can be characterized to the same extent or level (e.g., a live virus vaccine vs. a recombinant protein vaccine), and statistical tools are often used to determine alert or action levels in surveillance programs. Exceeding these levels requires the manufacturer to evaluate the situation but does not necessarily signal product failure.

GMP manufacturing entails facility design, process development, quality systems, and manufacturing surveillance. Together these systems help the manufacturer to control the production of a vaccine. As noted, many types of vaccine are marketed, and each has its unique features and therefore requires different plans for each of the steps mentioned in this section.

SEED LOT SYSTEMS

Seed lots are the stocks of specific strains of bacteria, viruses, or biotechnology-engineered cells used to express vaccine antigens. All seed lots should be documented in terms of their isolation, derivation (or construction, in the case of recombinant vector or engineered cells), and passage history. The purpose of a seed-lot system, which typically includes master and working stock seeds, and associated master and working cell banks, is to help ensure the consistency of vaccine manufacturing. The use of master and working seed lots provides a method to limit the replication of the seed and to minimize the possibility of genetic variation.

A master seed lot is a physically homogeneous preparation derived from an original seed processed at one time and passaged for a limited number of times. The master seed lot is characterized for its biological, biochemical and genetic characteristics, and to ensure its purity, its freedom from adventitious agents, and its clinical ability to produce an effective vaccine.

Cultures from the working seed lot should have the same characteristics as the master seed lot from which they are derived. For influenza vaccines, which may be reformulated with new virus antigens each year, certified seed lots can be obtained from national regulatory agencies.

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A working seed lot is derived from the master seed within a limited number of passages. The working seed is tested to ensure its purity, freedom from adventitious agents, and biochemical properties. The working seed is used for production of vaccine without intervening passages.

Bacterial Vaccine Seed Lot System

In the bacterial seed lot system, a master seed is subcultured to produce a working seed one passage beyond the master seed. An aliquot of the working seed is then expanded to produce a vaccine lot. The strain(s) used for the master seed lots are identified by historical records that include information about their origin. Information about the bacterial seed lot system should include source, passage history, and raw materials to which it was exposed, with specific emphasis on raw materials of ruminant origin. Seeds should be stored at an appropriate temperature in more than one location within a facility or at a distant site in order to decrease catastrophic risk.

Identity tests may include inoculation onto suitable biochemical media, Gram stains, genotype, and serological identification with suitable specific antisera. Special tests may be added, for example, to show culture viability but also lack of virulence.

Purity of the bacterial strains used for seed lots is verified by methods of suitable sensitivity to ensure that no adventitious agents are present. These purity tests often are performed in the presence of the seed under conditions where growth is inhibited by the presence or the absence of specific nutrients. Streaking can also be used to show that the cultured seed is a pure culture.

Viral Vaccine Seed Lot System

The derivation and passage history of viral seeds should be recorded in detail. Any manipulation of the viral phenotype (e.g., cold adaptation, development of temperature sensitivity, or attenuation of virulence) or intentional genetic manipulations (e.g., reassortment or recombination) should be documented.

These viral seeds are commonly differentiated into a master viral seed and working viral seeds or working viral stock. Viral seeds should be stored at cryogenic temperatures to promote stability and in more than one location within a facility or at a distant site to decrease catastrophic risk. Manufacturers should assess the following characteristics of the viral seed stock:

- Growth characteristics on the intended production cell substrate,
- Tissue tropism;
- Genetic markers;
- Identity (for recombinant vectors);
- Viability during storage,
- Genetic stability through production;
- Attenuation properties;
- Purity;
- Absence of adventitious agents. If attenuation or derivation is achieved by passage through different species, the viral seed should be assessed for absence of adventitious agents common to those species.

The master viral seed should be extensively characterized to demonstrate the stability of genotype and phenotype for a number of passages beyond the level used in production. Generally, during assessment of genetic stability, a master seed undergoes a minimum of five passes beyond the passage that will produce the final vaccine.

Tests should be performed for identity (e.g., sequencing the entire virus or a portion of it), adventitious agents, viral phenotype, genetic stability, and, if applicable, agents that might be present in the seed as a result of its passage history. Viral phenotype can be assessed further for tissue tropism, attenuation properties, and temperature sensitivity. Not all of these tests may be necessary for every viral seed strain.

In some cases the viral seeds may have a broad host range and therefore may require neutralization of the vaccine virus before they are tested for adventitious agent(s). If possible, testing for adventitious agents should be done without neutralization in order to avoid an antiserum that may inadvertently neutralize an adventitious agent present in the seed. Sometimes it is not possible to effectively neutralize a viral seed, and in such cases alternative strategies can be used. For example, the test can be performed in a cell substrate that does not permit replication by the vaccine virus. However, such a substitution of the substrate cell may compromise the test's sensitivity for detection of other adventitious agents. Therefore, the tests may be supplemented with use of polymerase chain reaction (PCR) assays.

Assessment of neurovirulence may be appropriate if the virus is known to be neurotropic. Manufacturers should consult with regulators about appropriate animal models, methods, and scoring systems for this assessment before they initiate such studies. For viruses that are neurovirulent or may revert to neurovirulence (e.g., polioviruses), it may be necessary to assess neurovirulence beyond the master seed.

If the master viral seed is well characterized, the working viral seed may not require extensive characterization. For example, it may not be necessary to repeat testing for all the relevant viruses from the derivation history.

Systems for Biotechnology-Engineered Vaccines

For a vaccine produced via a biotechnology-engineered cell-expression system, a master seed lot or a master cell bank will be established during product development. The seed lot or cell banks should be homogenous, which is often accomplished by limiting dilutions. The seed lot or cell bank system should be characterized in a manner analogous to that used for the cell substrate discussed in the next section, and additional tests can be used to demonstrate the genetic stability of the expression system.

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FERMENTATION AND CELL CULTURE MEDIA

A medium is the material in which an organism is grown and amplified in quantity to produce mass material for vaccine production. Its composition is diverse and depends on the cell types that the medium supports, ranging from well-defined chemical media to chemically undefined media that contain natural components such as sera from animal origin (see *Bovine Serum* ⟨1024⟩). Culture media should be suitable for their intended purpose and should be free from adventitious agents and known undesirable components such as toxins, allergens, and similar compounds. If undefined ingredients are necessary, the amount should be kept below levels that are demonstrated to be safe for the final product.

Fermentation Media for Bacterial Growth

The nutrients consist of materials like proteins, sugars, inorganic trace elements, amino acids, and vitamins needed for bacterial growth. The protein component may be as simple as free casein (milk protein), or it can be as complex as extracts from bacterial, plant, or animal sources. Any fermentation nutrients of animal origin are sourced carefully and tested for adventitious agents. The composition of a medium is often customized to optimize product quality attributes. Medium components that are known to cause allergic reactions should be avoided.

Media for Cell Culture for Viral Vaccines

The types and composition of media used for isolation and all subsequent culture of components of viral vaccines need to be recorded in detail. Chemically defined media without materials of animal origin are preferred. The medium should be tested for sterility and suitability for the cells used in product production. If materials of animal origin are used, they are assessed for freedom from adventitious agents. If human albumin is used in a U.S.-licensed vaccine, it must be licensed by FDA. The final product should be within specified limits of residual medium components such as serum, antibiotics, selection agents or reagents added for growth enhancement.

Media for Biotechnology-Engineered Cells

The requirement for media used for the fermentation and propagation of biotechnology-engineered cells is the same as that noted above for bacterial fermentation and cell culture growth.

PROPAGATION AND HARVEST

The propagation and harvest phases follow the manufacturing process from the initiation of cell growth in the working cell bank to the separation of the crude drug substance. In addition, in these manufacturing process steps, raw materials, media, and solutions should be qualified for their intended use. Batch numbers should be clearly assigned as needed, and the relationship between component harvests and batches of individual drug substances should be recorded clearly.

Propagation and Harvest for Bacterial Vaccines

Propagation of bacteria for bacterial vaccines is performed under specified conditions for the inoculum preparation and the fermentation phases. In-process monitoring and testing should be conducted for quality assurance. All controls and testing performed after production (e.g., purity, viability, antigen yield, and phenotypic identity) should be documented. The first step of drug-substance recovery is harvesting from the bioreactor. A variety of equipment is available, and the process equipment used depends on the nature of the process. Procedures should be established to ensure containment and prevention of contamination during harvesting and to monitor bioburden (including acceptance criteria) or sterility. The storage conditions and the stability time limit for the harvest material should be described. For most bacterial vaccines, an inactivation step is necessary. Personnel involved in bacterial inactivation should consider the following: how cell culture purity is verified after inactivation, whether culture purity should be defined before inactivation, choice of the inactivation agent, and validation of the procedure(s).

Propagation and Harvest for Viral Vaccines

The manufacturing of viral vaccines using eukaryotic cell culture includes a two-phase production process. The first is the expansion of the cell cultures used as a substrate for viral replication. The second phase includes the initial virus infection and subsequent replication and virus production.

CELL SUBSTRATE GROWTH PHASE

The cell substrate expansion process for viral production is the phase designed to prepare the cells in a physiological state appropriate to sustain virus growth. Cell substrates often require complex animal-derived supplements such as serum. The source and testing requirements of bovine serum are subject to regulatory requirements (see *Bovine Serum* ⟨1024⟩).

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VIRUS PRODUCTION PHASE

Relatively few cell types have been used as substrates in U.S.-licensed viral vaccines, but these include primary cells (e.g., certain cells derived from monkey, chick, or mouse tissue), diploid cell lines (e.g., WI-38, MRC-5, or FRhL-2), and continuous cell lines (e.g., Vero). Vaccine manufacturers have optimized nutrient requirements, growth factors, and serum concentration to support robust growth and strong virus productivity for these cell lines.

PURIFICATION

The objective of the purification steps is to remove as much as possible of the impurities in the initial harvest and to maximize the purity of the final vaccine product. Process residuals may consist of materials from the culture medium and/or cellular components. Purification procedures should be optimized and validated. When applicable, viral clearance steps (viral removal or inactivation) should be included and validated using relevant model viruses. Special considerations are observed depending on the types of vaccines and production system used, as discussed below.

Bacterial Fermentation

Bacterial fermentations are typically highly productive and yield large amounts of biomass. For bacterial subunit products or recombinant components expressed by bacteria, fermentation can produce very high concentrations of the desired active ingredient. Manufacturers should initiate culture purity testing before further processing.

LIVE BACTERIAL VACCINES

Live bacterial vaccines such as Bacillus Calmette-Guérin (BCG) and *Salmonella typhi* Ty21a are relatively fragile as pharmaceutical products and therefore tolerate only fairly gentle purification approaches. If osmotic and shear forces are constrained, then the integrity of the bacteria usually can be maintained.

INACTIVATED BACTERIAL VACCINES

At present no inactivated whole-cell bacterial vaccines are licensed for use in the U.S.

PURIFIED BACTERIAL ANTIGENS

Purification of bacterial components (e.g., proteins, toxins, and polysaccharides) generally requires cell disruption. More selective purification methods can be used to remove culture media and bacterial impurities and to achieve high purity of the target bacterial component.

Biotechnology-Engineered Cells

Of special concern in the purification of recombinant-derived vaccine components is the issue of residual host cell components that could produce an adverse immunogenic response in patients. This response could be exacerbated by the presence of vaccine adjuvants.

RECOMBINANT VIRUS-LIKE PARTICLES (VLP)

Formation of VLPs can coincidentally result in incorporation of host cell components (e.g., DNA) into the quaternary structure of the molecular assembly, resulting in a class of impurities that has a tight association with the active pharmaceutical ingredient. As a result, modern approaches to VLP production in some cases include a disassembly step that dissociates impurities from the viral proteins. This procedure is followed by a reassembly step that reforms the VLPs in the absence of the host components. Liquid-phase extractions and chromatographic procedures can be used to provide high-purity components for use in vaccine products with no substantial risk of carrying over significant residual host components.

Viral Vaccines Derived from Cell Culture

VIRAL VACCINES DERIVED FROM CONTINUOUS CELL LINES

If a continuous cell line (e.g., Vero) is used for vaccine production, a validated filtration step is necessary to separate virus from intact cells. The quantity and size of any residual host cell DNA also should be determined (see general information chapter *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩). Currently, 10 ng of host cell DNA is permitted per dose of a parenterally administered vaccine, and regulatory agencies continue to consider on a case-by-case basis the level of risk posed by host cell DNA for vaccines that are administered by other routes (e.g., nasal or oral). Multiple purification methods to reduce the size and amount of residual host-cell DNA present in the vaccine are desirable and include steps such as treatment with DNAse, diafiltration, ultrafiltration, and column chromatography.

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VIRAL VACCINES DERIVED FROM HUMAN DIPLOID CELL CULTURE

FDA has licensed several vaccines made using human diploid cells. The two most commonly used diploid cell lines are MRC-5 and WI-38, both of which are derived from human embryonic cells and have the normal diploid number of human chromosomes. They are widely used to manufacture vaccines because they have been shown to have no tumorigenic or oncogenic potential and have been shown to be susceptible to a wide range of human viruses. However, unlike continuous cell lines that can be passaged indefinitely, human diploid cell lines are capable of attaining only a certain number of population doublings, after which they experience a rapid decline in their ability to proliferate. This issue is managed by freezing multiple aliquots of master and working cell banks.

VIRAL VACCINES DERIVED FROM PRIMARY CELL CULTURE

Like diploid cells, primary cells normally are not tumorigenic or oncogenic. However, when primary cells are used to manufacture live vaccines, the donor animals from which the primary cells are obtained are extensively tested for a variety of pathogens before being used. For example, chicken flocks used to prepare chicken embryo kidney cells undergo extensive serological testing for adventitious agents before the flock can be used to prepare the cells. Some of these tests are described in the Code of Federal Regulations (CFR, see the sections listed in *Appendix 2*) and the USP general information chapter *Virology Test Methods* ⟨1237⟩.

Viral Vaccines Derived from Chicken Eggs

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. In the case of influenza vaccines, vaccine virus is harvested from egg allantoic fluid. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. Therefore, residual egg or embryo components are special considerations in vaccine purification.

Egg-based vaccine production, like all biomass expansions, requires care and quality control of the virus seed lots and egg substrates to avoid contamination with other organisms.

LIVE ATTENUATED VIRUS VACCINES

Viruses for live vaccines (e.g., yellow fever or live influenza) are produced using Specific Pathogen-Free (SPF) eggs. These eggs are produced by chicken flocks that are regularly screened for avian pathogens (e.g., avian leukosis virus) and are maintained using appropriate animal husbandry practices. To preserve the infectivity and antigenic integrity of the vaccine viruses while removing egg-derived components, relatively simple, mild methods (e.g., zonal sucrose gradient centrifugation and diafiltration) are used for vaccine virus concentration, purification, and buffer exchange.

INACTIVATED WHOLE VIRUS VACCINES

Viruses for inactivated vaccines can be produced using non-SPF eggs because of required chemical inactivation steps in the manufacturing process. Because the vaccine virus needs to be retained intact while removing egg-derived components and inactivating chemicals, relatively mild purification and concentration methods (e.g., zonal sucrose gradient centrifugation) are used. If chemical agents are used in the process, they should be minimized in the final product to below prespecified levels.

SPLIT VIRUS AND PURIFIED SUBUNIT VACCINES

Viruses for split virus and purified subunit influenza vaccines are produced in non-SPF embryonated eggs. Inactivation and purification of vaccine viruses are achieved by chemical treatment (e.g., formaldehyde or β-propiolactone) and zonal sucrose gradient centrifugation, respectively. Split virus vaccines are prepared by disruption of vaccine virus particles using a detergent (e.g., sodium deoxycholate) that preserves antigenic integrity.

INTERMEDIATES

Intermediates are defined here as the unformulated active (immunogenic) drug substances that are processed before final formulation and can be stored for long periods of time before further processing. These intermediates can be stored and should be included in a formal stability program. Examples of intermediates include bulk polysaccharides, purified recombinant proteins (concentrates), and conjugates.

Production of Intermediates

Intermediates are manufactured from starting materials by one or a combination of different processes (e.g., fermentation, cultivation, isolation, or synthesis). Subsequent steps of the procedure involve preparation, characterization, and purification, eventually resulting in the drug substance. Quality systems documents are adopted for production and all applicable information should be recorded in a controlled document (i.e., a batch record). When applicable, stability studies and release tests should be performed before proceeding to the next steps (see below).

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Tests for Intermediates

The quality attributes of the intermediate are commonly tested in conjunction with further processing. Characterization beyond release testing should be considered. Characterization methods can use appropriately qualified procedures. Some tests are routinely performed before the intermediates are converted to the final bulk, depending on individual vaccines.

If intermediates need to be stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays.

FINAL BULK

Final bulk is the bulk drug product that contains the drug substance(s), excipients, and other ingredients at desired concentrations and is ready for filling into individual containers.

Production of Final Bulk

Appropriately controlled amounts of all ingredients are blended to uniformity to produce the final bulk. The processing may include one or more steps such as buffer exchange and addition of diluents, bulking agent, stabilizing excipients, adjuvants, and preservatives. Final bulk may be prepared aseptically or processing may include a sterilization step.

Tests for Final Bulk

The quality attributes of the final bulk should be tested. Appropriate testing should be performed with respect to identity, purity, potency, sterility (see *Sterility Tests* ⟨71⟩), and antimicrobial effectiveness (see *Antimicrobial Effectiveness Testing* ⟨51⟩). Tests demonstrating safety, if applicable, are performed. The list includes, for example, tests for the absence of adventitious agents, mycoplasma, and other microorganisms.

Testing is required for specific process-related and product-related impurities, depending on the vaccines being manufactured. In addition, tests are required for the bulking agent, stabilizing excipients, adjuvants, and/or preservatives, if used. All the testing should be done according to respective standard operating procedures (SOPs), and all tests should have specifications (or provisional specifications, where applicable).

Stability Test for Final Bulk

If final bulks are stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays. Implementation of a stability program is required for formal stability studies, and the studies should be executed according to a protocol that contains detailed information about types of tests, including specifications, testing intervals, and data and analysis.

FINAL CONTAINER

A final container of vaccine contains the active ingredient(s) (i.e., antigen(s)) as well as additional components, such as stabilizers, adjuvants, or antimicrobial preservatives. They also may include residual materials from the manufacturing process.

Excipients and Other Additives

In addition to specific antigens, vaccines often include excipients and other additives that are intentionally added to the vaccine by the manufacturer for a specific purpose. These include adjuvants, antimicrobial preservatives, and stabilizers. Vaccines also contain manufacturing residuals, which are trace amounts of various components used during manufacturing. Thus, the combinations of these components comprise and define the complete vaccine product. Manufacturers must adhere to regulations governing permissible limits of such components, as indicated in the product's license.

ADJUVANTS

Adjuvants are agents incorporated into vaccine formulations to enhance and increase the immune responses generated by the vaccine antigens. Specifically, they can increase the amount of antibody produced, direct the immune response (Th1 or Th2), increase the duration of antibody presence (persistence), or produce a combination of these effects.

Aluminum compounds have long been the most widely used adjuvants worldwide. Two methods traditionally have been used for combining aluminum adjuvant to antigen to form aluminum-adsorbed vaccines. The first involves the addition of the antigen solution to preformed aluminum precipitate. The second involves the addition of an antigen to aluminum in solution and the addition of a compound that will coprecipitate the aluminum salt and the antigen in situ. Solutions of aluminum potassium sulfate, known as alum or aluminum chloride, have been used together with phosphate salts as precipitating agents. A number of aluminum adjuvant formulations are used in vaccines.

Tests for aluminum are based on metal detection tests described in the general test chapter *Aluminum* ⟨206⟩. Regulations limit the amount of aluminum permitted in a dose of vaccine. The Code of Federal Regulations [21 CFR 610.15(a), *Ingredients, preservatives, diluents, adjuvants*] states that "the amount of aluminum in the recommended individual dose of a biological product shall not exceed:

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1. 0.85 milligrams if determined by assay;
2. 1.14 milligrams if determined by calculation on the basis of the amount of aluminum compound added; or
3. 1.25 milligrams determined by assay provided that the data demonstrating that the amount of aluminum used is safe and necessary to produce the intended effect and are submitted to and approved by the Director, CBER [Center for Biologics Evaluation and Research at FDA]."

The third criterion above aligns U.S. regulations with World Health Organization guidance for aluminum content in a single human dose of a vaccine product.

Note that adjuvants are not licensed by themselves; they do not constitute a product. Rather, a vaccine consisting of specific antigen(s) and an adjuvant are licensed together as a drug product.

ANTIMICROBIAL PRESERVATIVES

In the case of multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeated puncture of multidose vials. With certain exceptions, a preservative is required to be present in vaccines marketed in multidose containers [21 CFR 610.15(a)]. Exceptions include yellow-fever vaccine; measles, mumps, and rubella (MMR); and dried vaccines when the accompanying diluent contains a preservative.

The microbial preservatives currently used in vaccines are thimerosal, 2-phenoxyethanol, benzethonium chloride, and phenol. These agents must pass the appropriate antimicrobial effectiveness test, as described in *Antimicrobial Effectiveness Testing* ⟨51⟩. Antimicrobial test challenges should be conducted as part of the normal formal stability program, including at expiration date. Various tests for preservatives can be found in *Antimicrobial Agents—Content* ⟨341⟩.

STABILIZERS

The primary purpose of stabilizers is to protect certain vaccines from adverse conditions such as heat or to serve as a cryopreservative during the lyophilization process, usually the freezing step. The particular materials chosen for this purpose include sugars (e.g., sucrose or lactose), amino acids (e.g., glycine or glutamic acid [monosodium salt]), glycerol, and proteins (e.g., human serum albumin [HSA] or gelatin). Materials should be customized to a specific vaccine formulation and selected with patient safety in mind.

When a protein is chosen as a stabilizer, two main safety concerns arise. One stems from the source of the protein: animal or human origin raises the possibility of the presence of an adventitious agent. The second concern is the possibility of an allergic reaction in persons sensitized to that protein. This should be evaluated as part of the clinical program during vaccine development. At present two proteins are used as stabilizers for vaccines: HSA and gelatin. FDA requires that any serum-derived albumin used in manufacturing be U.S.-licensed HSA. FDA guidance further recommends that a statement indicating the source and related risks appear in the "Warnings" section of the labeling for HSA-containing products.

Gelatin or processed gelatin also is used as a vaccine stabilizer. The gelatin source may be either bovine or porcine. Although the conditions of manufacturing gelatin are harsh (i.e., the product is subjected to extremes of heat and pH), there remains a concern with bovine sources about the presence of the transmissible spongiform encephalopathy (TSE) agent, because this agent is known to resist such conditions. Therefore, if gelatin added to a vaccine or used in manufacturing is from a bovine source, the material should have the appropriate documentation certifying that it comes from a country or region that is in compliance with TSE guidance for industry.

MANUFACTURING RESIDUALS

Vaccines may contain residual amounts of any of the materials used in the manufacturing process. These materials are termed manufacturing residuals. As a general principle, it is not possible to remove a particular substance completely, nor is it possible to conclusively demonstrate that a particular substance has been completely removed. Therefore the goal is to reduce these substances to an undetectable level, using a sensitive and validated analytical methodology. Some products are tested for pyrogenic substances as a manufacturing residual (see *Pyrogen Test* ⟨151⟩); and, if the product is freeze-dried, it should be tested for residual moisture (see *Loss on Drying* ⟨731⟩). Residual levels of manufacturing materials, including, if applicable, inactivating agents, should be justified. The release specifications of these components are required as part of the approved license.

CELL-DERIVED RESIDUALS

Live attenuated bacterial vaccines are not usually subject to a high degree of postexpansion purification. But killed bacterial component vaccines typically undergo significant purification to reduce cell-derived residuals. Common cellular components to be reduced are proteins, nucleic acids, and polysaccharides. Assays for these components are routinely conducted, if appropriate, to ensure purity. A common residual in bacterial vaccines made from Gram-negative bacteria is lipopolysaccharide (LPS), commonly known as endotoxin. Endotoxin testing is performed during the manufacturing process for any Gram-negative bacterial vaccine. In the case of Gram-positive bacterial vaccines, the endotoxin testing should be conducted to ensure that no contaminants from Gram-negative bacterial growth are present. Also, there must be a release specification for this residual. Two tests are currently used to detect LPS in biological products, the *Limulus* amebocyte lysate (LAL) test (see *Bacterial Endotoxins Test* ⟨85⟩) and the rabbit pyrogen test (see *Pyrogen Test* ⟨151⟩). The *Limulus* lysate that is used to test for bacterial endotoxin in FDA-regulated products is itself a U.S.-licensed product. The rabbit pyrogenicity test requires the use of animals and is more difficult to perform; therefore, it is not employed to the extent that the LAL test is used.

Viral vaccine manufacturing requires cell substrates to produce the viruses, which are then taken through purification processes. Generally, killed viral vaccines are more highly purified than are live attenuated ones. Depending on the method used to manufacture the vaccine, manufacturers work with FDA to develop prudent specifications for the final vaccine. Animal-derived host cells have been used extensively in vaccine manufacturing, particularly viral vaccines. For example, influenza and yellow fever vaccines are produced, respectively, in egg allantoic fluid and chicken embryos. Mumps, measles, and some

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rabies vaccines are produced in chick embryo cells. The labels of these products must state that residual chicken proteins may be present in the final vaccine, and the label may indicate how much is present. Further, the label also urges practitioner caution when vaccinating a person with known hypersensitivity to eggs.

Two U.S.-licensed hepatitis B vaccines are based on recombinant DNA-derived proteins expressed in yeast cultures. In both cases, the labels notify health care professionals that yeast protein may be present in the vaccine and recommend that suitable precautions should be exercised. In the case of live viral vaccines, considerations may be given to the reduction of cellular residual materials (e.g., host DNA, proteins).

MATERIALS OF ANIMAL ORIGIN

Some raw materials and reagents, such as gelatin, calf serum (see *Bovine Serum* ⟨1024⟩), or trypsin for vaccine manufacturing raise concerns regarding the potential presence of adventitious agents. Raw materials should be sourced from countries acceptable to FDA. Additionally, manufacturers should test these materials when possible to minimize the risks of contamination with adventitious agents. Reduction of serum components (e.g., BSA) should be considered in processing.

ANTIBIOTIC RESIDUALS

Some antibiotics (but not penicillin) can be used in minimal amounts in the manufacturing process for viral vaccines, according to 21 CFR 610.15(c). Those that have been used include gentamicin, streptomycin, neomycin, and polymyxin B. There is no requirement for tests of residual levels of these antibiotics in the final vaccine. However, according to 21 CFR 610.61(m), the calculated amount expected to remain as a residual in the final vaccine, based on the amount added and the dilution factor in the manufacturing process, must be stated on the product label.

INACTIVATING CHEMICAL AGENTS

Several chemical agents have been used to inactivate bacteria and viruses or to detoxify toxins in vaccine production processes. Formaldehyde and β-propiolactone are the most commonly used inactivating agents. Other less often used inactivating agents include glutaraldehyde and hydrogen peroxide. As a manufacturing residual, the inactivating agent should be removed from the final product as thoroughly as possible. The upper limit for formaldehyde is generally 0.02%, equivalent to 0.1 mg per 0.5-mL vaccine dose. The limit for β-propiolactone should be below the limit of detection.

EVALUATING THE STABILITY OF VACCINES

The stability of vaccine products depends on the nature of a vaccine antigen, the product formulation, and the control of vaccine storage prior to use.

Vaccine products are evaluated with programs that include real-time long-term storage under prescribed conditions. The use of extreme temperatures to potentially accelerate degradation may help manufacturers understand the stability of the product.

Vaccine products, like all pharmaceutical products, should be evaluated to define suitable conditions for storage (21 CFR 610.50 and 610.53). General principles of stability testing for biological products are described in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩. Typically these concerns are focused on the final vaccine product, but evaluations also are needed for bulk intermediates to justify the conditions under which they are held. In both cases manufacturers define in advance the conditions to which the product will be exposed (e.g., temperature, light, and humidity) and the time range during which the product will be exposed to those conditions. Stability studies should evaluate all storage conditions to which the product or intermediate is likely to be exposed during production, handling, shipping, and storage so that appropriate time limits can be placed on the exposure to those conditions.

The primary criteria for defining the storage conditions for these intermediates and the final products are generally focused on acceptable maintenance of potency; but, as discussed below, there often are other attributes that need to be considered.

Evaluation of the stability of vaccine products has three general purposes. First, the products are shown to maintain an acceptable analytical profile throughout manufacture and use to preserve safety and effectiveness. Second, stability studies across several product batches provide an effective way to characterize the inherent properties of the product. This in turn leads to the third use, demonstrating manufacturing consistency in the product.

Stability Protocols

The overall experimental plan for evaluating the stability profile of a given set of product or intermediate batches typically includes specific definition of the conditions under which the samples will be stored and why these conditions are relevant, the length of time the samples will be stored at each condition, when samples will be tested during this time course, and the analytical measurements at each time point. Additionally, these stability protocols include itemization of the analytical procedures to be used. For stability studies that occur early in product development, the studies may be conducted to confirm the suitability of the product formulation and/or storage conditions. Later in development, stability studies are typically conducted to provide data supporting product dating period or intermediate hold time, to provide more elaborate product characterization, and to evaluate manufacturing consistency. These latter studies define product end–expiry specifications that allow definitions of acceptable and unacceptable product. Unacceptable product is defined as product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency). Stability studies should be conducted over a duration sufficient to determine the point of loss of acceptable potency or other relevant parameters.

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Analytical Measurements

Manufacturers should consider the rigor of the analytical method(s) used to evaluate the stability of complex products and improve their understanding of the parameters that are critical to immunogenicity (including stability-indicator parameters). Selection of the stability-indicator parameters varies with each vaccine's unique characteristics.

The primary parameter that reflects stability for most vaccines is the potency assay (see *Potency Tests* in *Lot Release Testing*, below). This assay can take many forms, depending on individual vaccines (e.g., an infectivity assay for a live virus vaccine or a measure of the proportion of conjugated polysaccharide for a polysaccharide–protein conjugate vaccine). The potency assay is generally the key analytical result predicting whether a vaccine remains suitable for use and whether it will produce the expected clinical response. Other analytical measurements can provide important supplemental data, particularly those that have a clear link to the potency of the product. Examples include degradation profile, dissociation of a carrier protein from conjugated vaccines, and dissociation of an adjuvant from an antigen complex. Additionally, other common assays typically are performed as part of the stability study and may address physical or chemical changes in the product that may or may not affect its potency (e.g., general safety, degree of aggregation, pH, moisture, container, preservative, and enclosure).

Formal Evaluation of Stability Data and Product Expiry Dating

Vaccines must remain within potency specifications at the expiration date, provided that the product was stored under the normal conditions specified. Manufacturers should conduct stability studies to determine those storage conditions and that dating period to demonstrate that the product remains within the potency specifications. Manufacturers should conduct stability studies on a continuing basis. If a major manufacturing process changes, additional stability studies should be conducted to verify that there is no adverse impact on the stability profile. Under certain conditions such as process changes, accelerated stability studies could be conducted. An accelerated study involving temperatures both higher and lower than routine can evaluate the impact of temperature excursions on products. A similar evaluation should be done for product intermediates to establish how long a given intermediate can be held under defined conditions before it is processed further or discarded.

NOMENCLATURE

There are no uniform systems for naming new vaccines. 21 CFR 299 describes the cooperation of the FDA and the U.S. Adopted Names Council (USAN) in naming drugs, including vaccines. USAN is a private organization sponsored by the American Medical Association, USP, and the American Pharmacists Association. Section 262 in Title 42 of the Public Health Service Act requires that each package of the biological product be plainly marked with the proper name (name designated in the license 21 CFR 600.3) of the biological product contained in the package.

LABELING

Vaccine product labeling is regulated in compliance with 21 CFR 201 and 610. Requirements are set for container labeling and package labeling.

Container Label

Provisions are made for the following labels:

- Full label;
- Partial label; and
- No label on the container itself when the containers cannot support a label that includes all required information and should be placed in a package that does include all required information.

The label should be affixed to the container in a manner that allows visual inspection of the contents for the full length or circumference of the container. If no package exists, the container bears all of the information required for the package label.

The full container label normally contains the following:

- Proper name of the product;
- Name, address, and license number of the manufacturer;
- Lot number or other lot identification;
- Expiration date;
- Recommended individual dose, for multiple-dose containers;
- The phrase *Rx only* for prescription biologicals; and
- Any applicable cautionary statements.

Package Label

In addition to the information required on the container label, the package label should describe the following:

- Any preservative used and its concentration, or the words *no preservative* if no preservative is used and its absence is a safety factor;
- Number of containers, if more than one; or

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- Amount of product in the container, expressed as number of doses, volume, units of potency, weight, and equivalent volume (for dried product to be reconstituted); or
- A combination of the above to provide an accurate description of the contents, as applicable;
- Recommended storage temperature;
- The words *shake well, do not freeze,* or the equivalent, as well as other instructions when indicated by the character of the product;
- Recommended individual dose, for multiple-dose containers;
- Recommended route of administration, or reference to such directions in an enclosed circular;
- Presence of known sensitizing substances;
- Type of antibiotics added during manufacture and the amount calculated to remain in the final product;
- Inactive ingredients, when they constitute a safety factor or are referenced to an enclosed circular;
- Adjuvant, if present;
- Source of the product, when this may be a factor in safe administration;
- Identity of each microorganism used in manufacture and, if applicable, the production medium and the method of inactivation or reference to an enclosed circular;
- Minimum potency in terms of official standard of potency, or the words *no U.S. standard of potency.*

Prescribing Information

Detailed information about a vaccine appears in its prescribing information, commonly called the package insert. Increasingly, vaccines are distributed with patient package inserts written in lay language. Prescribing information (21 CFR 201.56 and 201.57) includes the following:

- Highlights of prescribing information
- Product names, other required information
- Boxed warning
- Recent major changes

1. Indications and usage
2. Dosage and administration
3. Dosage forms and strengths
4. Contraindications
5. Warnings and precautions
6. Adverse reactions
7. Drug interactions
8. Use in specific populations (e.g., pregnancy, nursing mothers, pediatric, geriatric)
9. Drug abuse and dependence
10. Overdosage
11. Description
12. Clinical pharmacology
13. Nonclinical toxicology
14. Clinical studies
15. References
16. How supplied/storage and handling
17. Patient counseling information

LOT RELEASE TESTING

General Principles

Manufacturers perform all appropriate tests for the licensed specifications for the product, according to 21 CFR 610.1 and 610.2. Samples of each licensed lot and protocols containing the manufacturers' test results are submitted to FDA. After FDA evaluates the protocol to ensure that the product specifications are met, and after satisfactory confirmatory testing, FDA approves the release of the lot if all tests meet the standards of safety, purity, and potency established for the particular vaccine product. After approval is granted, the manufacturer distributes and markets the product.

Guidelines are available regarding alternatives to lot release and a surveillance system. All of these variations are subject to the regulations in 21 CFR 610.2 that allow FDA to require that samples of any lot of licensed product (e.g., vaccine), together with the protocols showing results of applicable tests, be sent to FDA.

Common Tests

The tests common to all lots of all products include tests for potency, general safety, sterility, purity, identity, and constituent materials. The manufacturer completes these tests for conformity with standards applicable to each product. The results of all tests are considered, except when a test has been invalidated as a result of causes unrelated to the product (21 CFR 610.1).

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POTENCY TESTS (VACCINE-SPECIFIC)

The basic definition and requirements for vaccine potency and potency assays are provided in 21 CFR 600.3 and 610.10. A vaccine potency assay should indicate the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard. Product potency tests vary with vaccine product types (e.g., viral, bacterial, live attenuated, inactivated, or polysaccharide). As a result, potency assays for vaccines span a variety of approaches to the expression of potency. In vitro potency tests for live virus may include plaque formation assays, endpoint dilution assays (e.g., the tissue culture infective dose [$TCID_{50}$], virus neutralization assays, or quantitative polymerase chain reaction [PCR] assays). Quantitative colony formation assays are used for live attenuated bacterial vaccines. Animal challenge tests for immunogenicity assays of potency, such as those for diphtheria and tetanus (U.S. Department of Health, Education, and Welfare, 1953; see *Appendix 2*), or rabies and anthrax show in vivo response. Antigenicity assays use enzyme-linked immunosorbent assays (ELISA), e.g., with hepatitis A or rate nephelometry and rocket immunoelectrophoresis (e.g., with pneumococcal polysaccharides). The potency tests for bacterial vaccines, such as the meningococcal polysaccharides, pneumococcal polysaccharides, or *Haemophilus b* protein conjugate vaccines use chemical and physical chemical assays. In the case of pure polysaccharide vaccines, the concentration or quantity of the vaccine component (polysaccharide) and its quality (e.g., size) have been shown to be indicative of human immune response.

Assay precision and reproducibility vary with the different methodologies that are used in potency assays, ranging from the high accuracy and precision of chemical tests at one end of the spectrum to bioassays at the other end. The general test chapter *Design and Analysis of Biological Assays* ⟨111⟩ provides guidance for bioassays and applies to vaccine potency assays. Other tests should be validated as described in the general information chapter *Validation of Compendial Procedures* ⟨1225⟩.

RELEASE TESTS

Official release of vaccines by the vaccine regulatory authority may be based on either the bulk or the final container. It is highly desirable to perform potency tests on the final container. However, under certain circumstances this may not be practical or even possible: thus, a case-by-case approach would be required. The choice of whether to test the bulk or the final container derives from a number of considerations, such as the quantity of vaccine available for tests at the different manufacturing stages. For certain vaccines, both bulk and final container receive official release. The potency test is generally required for the final container. If it is not feasible to perform the potency test on the final drug product, the test is performed on the bulk material.

GENERAL SAFETY

For biological products that are intended for administration to humans, manufacturers perform a general safety test in order to detect any extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR 610.11.

STERILITY

A sterility test of each lot of each product is conducted according to procedures described in *Sterility Tests* ⟨71⟩ and 21 CFR 610.12 for both bulk and final container material.

BACTERIAL ENDOTOXINS

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins, as indicated in *Bacterial Endotoxins Test* ⟨85⟩.

PURITY

Vaccines need to be free of extraneous material. Approved vaccine license applications indicate extraneous materials that are unavoidable in the manufacturing process for a specific product. The application may indicate test results and allowable limits for such materials, according to procedures described in 21 CFR 610.13.

RESIDUAL MOISTURE

Each lot of dried product is tested for residual moisture [see 21 CFR 610.13 (a), *Loss on Drying* ⟨731⟩, and FDA's *Guideline for the Determination of Residual Moisture in Dried Biological Products* (see *Appendix 2*)].

PYROGENS

Each lot of final containers of a vaccine intended for use by injection is tested for pyrogenic substances, as indicated in *Pyrogen Test* ⟨151⟩ and 21 CFR 610.13 (b).

IDENTITY

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established by physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific cultural tests, or in vivo or in vitro immunological tests. In large part, identity tests are performed to distinguish the subject vaccine from other materials manufactured at the same site (21 CFR 610.14).

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CONSTITUENT MATERIALS

Ingredients, preservatives, diluents, adjuvants, extraneous protein, cell culture-produced vaccines, and antibiotics are tested according to 21 CFR 610.15.

Permissible Combinations

Formulations that combine several vaccines must be licensed as combinations (21 CFR 610.17). The potency of each vaccine in the combination is individually tested and must meet the specifications in the context of the final combined product; other appropriate quality tests apply as well. For vaccines that are physically combined in clinical locations just before administration to a patient, prescribing information should describe specific procedures to follow in those settings.

Quality

In general, quality control systems for vaccine manufacture are identical to those routinely employed for production of other pharmaceuticals. These include raw material testing and release, manufacturing, process-control documentation, and aseptic processing. Manufacturers formally assign responsibility to designated staff for maintaining the continued safety, purity, and potency of the product and for ensuring compliance with applicable product and establishment standards, along with compliance with current GMPs. Analysts use reference standards and validated methods to determine active ingredients, residuals, and impurities. Manufacturers determine product safety in a variety of ways that may include the use of experimental animals, procedures to demonstrate product sterility, and tests to ensure product potency. The complexity of the quality control systems for vaccines lies in the variety of methods used to produce and control production. Lot release testing proceeds according to 21 CFR 610.2 and involves evaluating lots for safety, purity, and potency before release. Manufacturers follow FDA and applicable international standards for testing and validation. The basic considerations for validation are included in *Validation of Compendial Procedures* ⟨1225⟩, in addition to guidance documents issued by FDA and the International Conference on Harmonization (ICH) (see *Appendix 2*).

Alternative Tests

Modification of test methods or manufacturing processes as licensed may be permitted if the regulatory authority can be assured that the modifications cause no reduction in safety, purity, potency, and effectiveness of the biological product. It may be necessary for the manufacturer to file the proposed changes prior to implementation (21 CFR 601.12 and 21 CFR 610.9).

GLOSSARY

Acceptance criteria: The product specifications and acceptance or rejection criteria, with an associated sampling plan, necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units).

Active ingredient: Any component intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or other animals. The term includes those components that may undergo chemical change in the manufacture of the drug product and may be present in the drug product in a modified form intended to furnish the specified activity or effect.

Adventitious agent: A microorganism (e.g., bacteria, fungi, mycoplasma, spiroplasma, mycobacteria, rickettsia, viruses, protozoa, parasites, TSE agent) that is inadvertently introduced into the production of a biological product.

Batch: A specific quantity of a drug or other material intended to have uniform character and quality, within specified limits, and produced according to a single manufacturing order during the same cycle of manufacture.

Biological product: Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man.

Cell bank: Vials of cells of uniform composition (not necessarily clonal) derived from a single tissue or cell, aliquoted into appropriate storage containers, and stored under appropriate conditions.

Cell line: Cells that have been propagated in culture since establishment of a primary culture and have survived through crisis and senescence. Such surviving cells are immortal and will not senesce. Diploid cell strains have been established from primary cultures and expanded into cell banks, but have not passed through crisis and are not immortal.

Characterization: Determination of the properties of a substance.

Component: Any ingredient intended for use in the manufacture of a drug product, including those that may not appear in such drug product.

Container (also final container): The immediate unit, bottle, vial, ampule, tube, or other receptacle containing the product as distributed for sale, barter, or exchange.

Control: Having responsibility for maintaining the continued safety, purity, and potency of the product and for compliance with applicable product and establishment standards, and for compliance with current good manufacturing practices.

Control cells: Cells that are split off from the production culture and maintained in parallel under the same conditions and using the same reagents (e.g., culture medium) to perform quality control tests on cells that have not been exposed to the vaccine virus (which may interfere with some tests).

Dating period: The period beyond which the product cannot be expected beyond reasonable doubt to yield its specific results.

Diploid: Having the expected number of chromosomes for a species (i.e., two of each autosomal chromosome and two sex chromosomes).

Drug product: A finished dosage form (e.g., solution, suspension) that contains an active drug ingredient generally in association with inactive ingredients.

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End-of-production cells: Cells harvested at the end of a production run or cells cultured from the master cell bank or working cell bank to a passage level or population doubling level comparable to or beyond the highest level reached in production.

End-of-production passage level: The maximal passage level achieved during manufacturing at final vaccine harvest. Cells may be evaluated at this level or beyond.

Endogenous virus: A virus whose genome is present in an integrated form in a cell substrate by heredity. Endogenous viral sequences may or may not encode for an intact or infectious virus.

Expiration date: The calendar month and year, and where applicable, the day and hour, that the dating period ends.

Filling: A group of final containers identical in all respects, which have been filled with the same product from the same bulk lot without any change that will affect the integrity of the filling assembly.

Final bulk: The stage of vaccine production directly prior to filling of individual vials.

Free of and freedom from: For a substance to be considered free of a contaminant, an assay must demonstrate that a defined quantity of the substance is negative for that contaminant to a defined level of sensitivity. The level of assay sensitivity is defined by the choice of assay and can be determined experimentally using standardized reagents. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate freedom from that contaminant.

Harvest: Collection of material at the end of vaccine virus propagation in cell culture, from which vaccine will be prepared. This material may be the culture supernatant, the cells themselves (often in disrupted form), or some combination thereof.

Inactive ingredient: Any component other than an active ingredient.

In-process material: Any material fabricated, compounded, blended, or derived by chemical reaction that is produced for, and used in, the preparation of the drug product.

Intermediates: Unformulated active ingredients that are processed before final formulation and can be stored for long periods of time before further processing.

Label: Any written, printed, or graphic matter on the container or package or any such matter clearly visible through the immediate carton, receptacle, or wrapper.

Latent virus: A virus that is present in a cell, without evidence of active replication, but with the potential to reactivate, is considered to be microbiologically latent.

Lot: A batch, or a specific identified portion of a batch, having uniform character and quality within specified limits; or, in the case of a drug product produced by continuous process, it is a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits.

Lot number, control number, or batch number: Any distinctive combination of letters, numbers, or symbols, or any combination of them, from which the complete history of the manufacture, processing, packing, holding, and distribution of a batch or lot of drug product or other material can be determined.

Manufacture: All steps in the propagation or manufacture and preparation of products. Includes, but is not limited to, filling, testing, labeling, packaging, quality control, and storage by the manufacturer.

Manufacturer: Any legal person or entity engaged in the manufacture of a product subject to license under the Public Health Service (PHS) Act. Manufacturer also includes any legal person or entity who is an applicant for a license where the applicant assumes responsibility for compliance with the applicable product and establishment standards.

Master cell bank: A bank of a cell substrate from which all subsequent cell banks used for vaccine production will be derived. The master cell bank represents a characterized collection of cells derived from a single tissue or cell.

Master virus seed: A viral seed of a selected vaccine virus from which all future vaccine production will be derived, either directly, or via working virus seeds.

Oncogenicity: The property of certain biological agents (e.g., viruses) or materials (e.g., nucleic acids) that are capable of immortalizing cells and endowing them with the capacity to form tumors. Oncogenicity is distinct from tumorigenicity.

Package: The immediate carton, receptacle, or wrapper, including all labeling matter therein and thereon, and the contents of the one or more enclosed containers. If no package is used, the container shall be deemed to be the package.

Passage level: The number of times, since establishment from a primary cell culture, a culture has been split or reseeded.

Population doubling level: The number of times, since establishment from a primary cell culture, a culture has doubled in number of cells.

Potency: The therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard.

Primary cells: Cells placed into culture immediately after an embryo, tissue, or organ is removed from an animal or human and homogenized, minced, or otherwise separated into a suspension of cells. Primary cells may be maintained in medium, but are not passaged (split).

Process: A manufacturing step that is performed on the product itself which may affect its safety, purity, or potency, in contrast to such manufacturing steps which do not affect intrinsically the safety, purity, or potency of the product.

Proper name: The name, designated in the license, to be used on each package of the product.

Purity: Relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product. Purity includes but is not limited to relative freedom from residual moisture or other volatile substances and pyrogenic substances.

Qualification: Determination of the suitability of a material for manufacturing based on its characterization.

Safety: The relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time.

Specification: The quality standard (i.e., tests, analytical procedures, acceptance criteria) provided in an approved application to confirm the quality of products, intermediates, raw materials, reagents, components, in-process materials, container–closure systems, and other materials used in the production of a product.

Standards: Specifications and procedures applicable to an establishment or to the manufacture or release of products, which are prescribed in this subchapter or established in the biologics license application and designed to ensure the continued safety, purity, and potency of such products.

Sterility: Freedom from viable contaminating microorganisms, as determined by tests prescribed by the FDA.

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Tumorigenic: A property of certain cell types to form tumors when inoculated into animals (generally a syngeneic, an immunosuppressed allogeneic, or an immunosuppressed xenogeneic host). These tumors may be at the injection site or a different site and may also metastasize to other sites.

Tumorigenicity: The process by which immortalized cells form tumors when inoculated into animals. Tumorigenicity is distinct from oncogenicity.

Unacceptable product: Product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency).

Validation: The performance characteristics of an analytical procedure, based on the demonstration that the procedure is suitable for its intended purpose or use. Validation of a process is the determination of the extent to which a process meets the requirements for the various performance characteristics and the demonstration that the process uniformly performs to defined characteristics. Validation is generally performed in accordance with *Validation of Compendial Procedures* ⟨1225⟩ and the relevant ICH guidelines.

Viral clearance: The combination of the physical removal of viral particles and the reduction of viral infectivity through inactivation.

Virus seed or viral seed: A live viral preparation of uniform composition (not necessarily clonal) derived from a single culture process, aliquoted into appropriate storage containers, and stored under appropriate conditions.

Working cell bank: A cell bank derived by propagation of cells from the master cell bank under defined conditions and used to initiate production cell cultures on a lot-by-lot basis.

Working virus seed: A viral seed derived by propagation of virus from the master virus seed under defined conditions and used to initiate production cell cultures lot-by-lot.

APPENDICES

Appendix 1: Types of Vaccines Currently Licensed in the U.S. (examples)

- Bacterial, live attenuated (e.g., *Salmonella typhi*)
- Bacterial, polysaccharide (e.g., meningococcal, pneumococcal)
- Bacterial, polysaccharide-protein conjugate (e.g., meningococcal, pneumococcal)
- Bacterial, toxoid (e.g., diphtheria, tetanus)
- Bacterial, extracts (e.g., pertussis, anthrax)
- Viral, live attenuated (e.g., influenza, measles, mumps, rubella)
- Viral, whole inactivated (e.g., rabies)
- Viral, subunit (e.g., influenza, hepatitis B, human papillomavirus)

Appendix 2: Selected Regulatory Documents

- 21 CFR 201.
- 21 CFR 299.
- 21 CFR 600.
- 21 CFR 610.
- Section 262 in Title 42 of the Public Health Service Act
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guidance for Industry—Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by Blood and Blood Products* (January 2002). http://www.fda.gov
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guideline for the Determination of Residual Moisture in Dried Biological Products* (January 1990). http://www.fda.gov
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guidance on Alternatives to Lot Release for Licensed Biological Products.* Federal Register 1993;58(137): 38771–38773. http://www.fda.gov
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guidance For Industry—Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases* (September 2006). http://www.fda.gov
- FDA periodically issues or updates Guidance for Industry and posts these documents at http://www.fda.gov/cber/guidelines.htm
- Department of Health, Education, and Welfare (now the National Institutes of Health). *Minimum Requirements for Immune Serum Globulin (Human).* 3rd rev. Bethesda, MD: Department of Health, Education, and Welfare, 1953.
- International Conference on Harmonization (ICH). Q2(R1). *Validation of Analytical Procedures: Text and Methodology* at http://www.ich.org

Add the following:

▲⟨1236⟩ SOLUBILITY MEASUREMENTS

INTRODUCTION

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BACKGROUND
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Methods of Estimating Aqueous Solubility
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EXPERIMENTAL METHODS
Methods for Determination of Equilibrium Solubility
METHODS FOR DETERMINATION OF APPARENT SOLUBILITY
Intrinsic Determination (Rotating Disk)
Potentiometric Titration
Turbidimetry
Physical Assessment of Solubility
SOLUBILITY MEASUREMENTS IN BIORELEVANT MEDIA
Human Fasted-State Simulated Gastric Fluid (FaSSGF)
Human Fed-State Simulated Gastric Fluid (FeSSGF)
Human Fasted-State Simulated Intestinal Fluid (FaSSIF-V2)
Human Fed-State Simulated Intestinal Fluid (FeSSIF-V2)
Human Simulated Colonic Fluid—Proximal Colon (SCoF2)
Human Simulated Colonic Fluid—Distal Colon (SCoF1)
Canine Fasted-State Simulated Gastric Fluid (FaSSGFc pH 1.2–2.5)
Canine Fasted-State Simulated Gastric Fluid (FaSSGFc pH 2.5–6.5)
Canine Fasted-State Simulated Intestinal Fluid (FaSSIFc)
Bovine Simulated Ruminal Fluids
GLOSSARY
REFERENCES

INTRODUCTION[1]

Solutes may differ in both the extent and the rate at which they dissolve in a solvent. Solubility is the capacity of the solvent to dissolve a solute whereas dissolution rate is how quickly the solubility limit is reached. Equilibrium solubility is the concentration limit, at thermodynamic equilibrium, to which a solute may be uniformly dissolved into a solvent when excess solid is present. The apparent solubility may be either higher or lower than the equilibrium solubility due to transient supersaturation or incomplete dissolution due to insufficient time to reach equilibrium. Equilibrium can be defined as sufficiently converged when it no longer changes significantly during a certain time frame. Solubility may be stated in units of concentration such as molality, molarity, mole fraction, mole ratio, weight/volume, or weight/weight.

Solubility can be expressed in absolute as well as relative terms. One method of describing the absolute solubility is the descriptive solubility defined in *General Notices, 5.30 Description and Solubility*. Relative measures of the solubility are important for predicting the drug delivery characteristics of a dosage form and characterizing a drug as either high solubility or low solubility in the biopharmaceutics classification system (BCS) (*1*).

Accurate determination of the solubility of pharmaceutical materials is important for understanding both quality control and drug delivery issues for pharmaceutical formulations. The apparent solubility (see the *Glossary*) of a material is affected by the physicochemical properties of the material (e.g., surface area, particle size, crystal form), the properties of the solubility media (e.g., pH, polarity, surface tension, added surfactants, co-solvents, salts), and the control of the solubility measurement parameters (e.g., temperature, time, agitation method). Additionally, the apparent solubility may be comprised of the intrinsic solubility of the uncharged moiety, the solubility of the ionized compound, and the effect of solubilizers and multiple crystal forms or salt forms. Control of these experimental factors during solubility measurements is key to obtaining accurate, reliable values for the equilibrium solubility of a material.

This chapter will begin with a discussion of the concepts and equations that are relevant to solubility measurements. Understanding these relationships is fundamental to accurate evaluation of solubility. This will be followed by a brief description of typical experimental methods used to assess solubility of pharmaceutical materials. Finally, the use of solubility measurements to obtain biorelevant solubility (for human products) and species-dependent solubility (for veterinary products) will be discussed.

BACKGROUND

Thermodynamic Equilibrium and Solubility

Dissolution of a crystalline solid solute can be modeled by the two-step process of melting the crystal into a pure liquid solute followed by mixing the liquid solute into the solvent. The Gibbs free energy of mixing determines whether, and to what extent, two compounds mix to form a homogeneous phase.

[1] The terms used in this chapter are defined in the *Glossary*.

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$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix}$$

ΔG_{mix} = Gibbs free energy of mixing
ΔH_{mix} = enthalpy of mixing; indicates if the mixing is an endothermic or exothermic process
T = temperature in degrees Kelvin
ΔS_{mix} = change in entropy (disorder) that results from mixing

If the change in the Gibbs free energy is negative, the mixing will be thermodynamically favored. When equilibrium is reached, ΔG will be equal to zero. The enthalpy of mixing is due to the breaking of cohesive interactions (solute–solute, solvent–solvent) and the creation of adhesive interactions (solute–solvent). In other words:

$$\Delta H_{mix} = H_{UU} + H_{VV} - H_{UV}$$

ΔH_{mix} = enthalpy of mixing
U = solute
V = solvent

This enthalpy change is also equivalent to the work done by removing a volume of pure solvent and a volume of pure solute and exchanging them. The enthalpy change is equal to the newly created interfacial surface energy according to:

$$\Delta H_{mix} = \gamma_{UV}A_U = \sum_i \gamma_{iV}A_i$$

γ_{UV} = solute-solvent interfacial surface tension
A_U = interfacial surface area
γ_{iV} = group-solvent interfacial surface tension for group i
A_i = surface area of group i

This equation also shows how the total surface energy can be broken into i smaller groups each with its own surface area, A_i, and corresponding group-water interfacial tension γ_{iV}.

For an ideal system, ΔH_{mix} is zero because the interactions between the ideal solute and ideal solvent are identical. The entropy of mixing for an ideal solution always increases as a result of mixing and is given by:

$$\Delta S_{mix}^{ideal} = R(X_U \ln X_U + X_V \ln X_V)$$

which under dilute conditions can be simplified to:

$$\Delta S_{mix}^{ideal} = R \ln X_U$$

For an ideal system under dilute conditions when a crystalline solid is in equilibrium with a saturated solution of the same solute:

$$\Delta G_U^{ideal} = -T\Delta S_U^{ideal} = -RT \ln X_U$$

$$\ln X_U = \frac{\Delta G_m}{RT}$$

R = gas constant
X_U = concentration of the solute expressed as a mole fraction
ΔG_m = the free energy change from melting the crystalline solid

Which illustrates how the solubility of the substance can be correlated with the melting point. For a real solution, the solute may also affect (reduce) the disorder in the solvent by inducing structure to the solvent. Therefore, for a real solution, this yields:

$$\Delta G_{mix}^{real} = \sum_i \gamma_{iV}A_i - T(\sum_i h_i A_i + \Delta S_{mix}^{ideal})$$

h_i = entropic effect on solvent of group i with area A_i

This model allows the total solute surface area (A_U) to be broken into smaller pieces ($\sum_{Ai}$) and the contribution of these groups to the enthalpic and entropic contributions to the free energy can be estimated.

Methods of Estimating Aqueous Solubility

Yalkowsky demonstrated (*2,3*) that a relatively simple general solubility equation (GSE) may be used to empirically estimate the intrinsic solubility of compounds in water.

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$$\log S_0 = 0.5 - 0.01(MP - 25) - \log K_{OW}$$

S_0 = intrinsic solubility (of the unionized molecule)
MP = melting point of the crystalline solid (in degrees Celsius)
K_{OW} = octanol–water partition coefficient; the temperature of the water is 25°

The GSE indicates that the aqueous solubility will be reduced for compounds with a higher melting point and compounds with a higher tendency to partition into an oil phase (octanol). The logarithm of the octanol–water partition in the GSE accounts for the difference between an ideal solution and an aqueous solution due to the enthalpy of mixing (*3*). The GSE can also be used to predict the solubility of ionizable compounds by combining it with the Henderson–Hasselbalch equation if the pK_a is known (see *Effect of pH*).

Use of GSE requires the measurement of the melting point and partition coefficient (and pK_a for ionizable compounds). There are several computer programs that will support the estimation of the partition coefficient and the pK_a for compounds based on structure (*4*), but this is not the case for melting points. Efforts to develop computational methods to predict aqueous solubilities have relied on training sets of molecules to search for correlations with properties that can be more easily predicted from the structure (e.g., molecular weight, solvent-accessible surface area, number of rotatable bonds, etc.) (*5*). The success of these computational approaches is often limited to molecules that are similar to the training set. These calculational methods are adequate for providing assistance in prescreening synthetic candidates, but are not sufficiently accurate to substitute for experimental solubility.

Factors that Affect Solubility and Solubility Measurements

EFFECT OF pH

The solubility of ionizable acids and bases is pH dependent because the charged species have a higher affinity for the aqueous environment than the neutral form. The total solubility of the ionizable acid or base is the sum of the intrinsic solubility and the amount of ionized solute present at that pH. The Henderson–Hasselbalch equation relates the increase in the solubility to the pH of the solution relative to the pK_a (acidic) or pK_a (basic) of the ionizable acid or base.

$$\text{pH} = \text{p}K_a + \log\frac{[A^-]}{[HA]};\ S_{tot} = S_0\left(1 + 10^{(\text{pH} - \text{p}K_a)}\right)$$

pK_a = $-\log(K_a)$
K_a = acid dissociation constant
$[A^-]$ = molar concentration of the acid's conjugate base
$[HA]$ = molar concentration of the undissociated weak acid
S_{tot} = total solubility of weak acid
S_0 = intrinsic solubility of uncharged moiety

$$\text{pH} = \text{p}K_a + \log\frac{[B]}{[BH^+]};\ S_{tot} = S_0\left(1 + 10^{(\text{p}K_a - \text{pH})}\right)$$

pK_a = $-\log(K_a)$
K_a = base dissociation constant
$[B]$ = molar concentration of the base's conjugate base
$[BH^+]$ = molar concentration of the dissociated base
S_{tot} = total solubility of weak base
S_0 = intrinsic solubility of uncharged moiety

The Henderson–Hasselbalch equation helps explain the increase in solubility at the first pK_a, but is not useful for modeling the behavior of polyprotic acids over a pH range incorporating additional pK_a values. Because ionizable molecules can differ in the number and type of ionizable groups, it is important to explore solubility across a range of pH values. *Figure 1* illustrates this pH dependence of the solubility for a molecule with two ionization constants of 5.6 and 11.7. The molecule is charged below pH 5.6 and above 11.7 and is neutral between these two pH values. Where the molecule is unionized, the solubility is equal to the intrinsic solubility. For the ionized molecule, the solubility increases on a log scale as the pH changes. The formation of a salt may limit the solubility at a low or high pH (see *Figure 1*). If the acid used to adjust the pH contributes the counter-ion for the salt, the common-ion effect will further suppress the solubility as the counter-ion concentration is increased (see *Figure 1*). If the salt is dissolved at higher pH, the salt may supersaturate the solution initially, but will eventually precipitate as whatever solid form has lower solubility at that pH (*6*).

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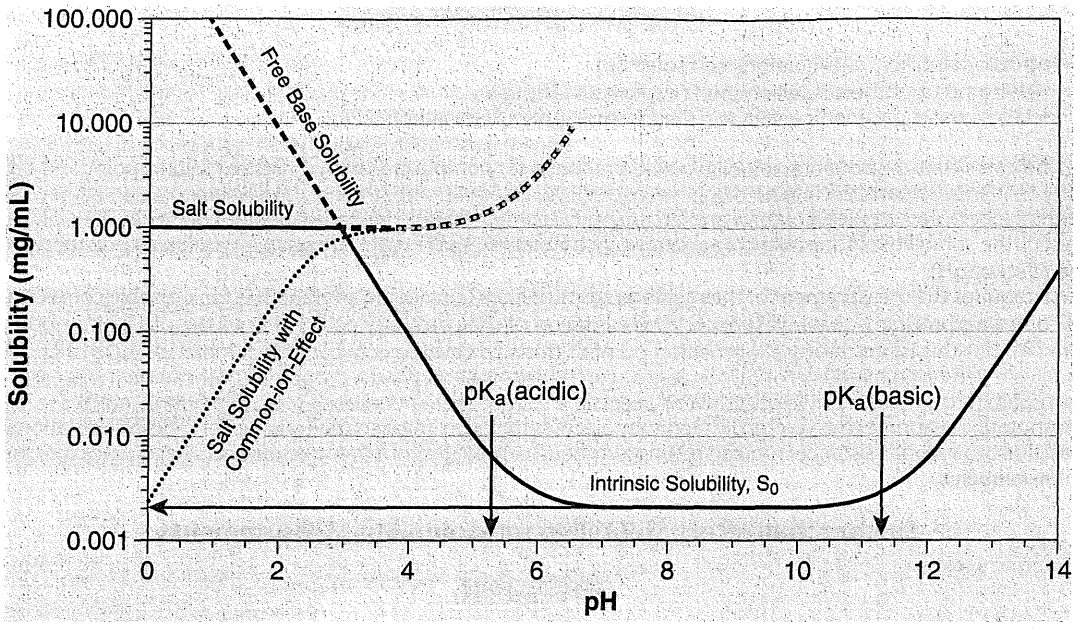

Figure 1. Effect of pH on solubility for an ionizable compound. When the molecule is unionized, the solubility is equal to the intrinsic solubility. For the ionized molecule, the solubility increases on a log scale as the pH changes. The salt solubility limits the solubility at a low pH. If the acid used to adjust the pH contributes the counter-ion for the salt, the common-ion effect will further suppress the solubility as the counter-ion concentration is increased (apparent at pH <2 in the figure).

EFFECT OF SALTS AND COUNTER-IONS

Ionizable compounds can also form salts with an oppositely charged counter-ion (*6*). In a solution, in the presence of the charged counter-ion, the solubility product describes this equilibrium reaction as follows:

For the salt dissolution, $A_nB_m(s) \rightarrow nA^+(aq) + mB^-(aq)$
solubility product, $K_{sp} = [A^+]^n[B^-]^m$

The maximum solubility of the salt in a solution is also illustrated in *Figure 1*. As a result of the formation of the salt, the actual solubility of the charged molecule is seen to plateau (at a pH below the pK_a of the drug in this example) instead of continuing to increase as predicted by the Henderson–Hasselbalch equation. Because the solubility product, K_{sp}, is a constant, the solubility of the ionizable moiety may drop even further if the acid being used to adjust the pH increases the concentration of the oppositely charged counter-ion. The reduction in the solubility of the charged molecule as the counter-ion concentration is increased is referred to as the common-ion effect (*6*). This is frequently seen when hydrochloric acid (HCl) is used to reduce the pH, and the solubility of the chloride salt is reduced due to increasing chloride concentration (e.g., at pH <2). Although not illustrated in *Figure 1*, salts may also limit the solubility on the basic side of the plot (e.g., a sodium salt of an acid moiety), and the common-ion effect may similarly affect the solubility at a high pH when the compound used to adjust the pH has a common-ion (e.g., sodium hydroxide).

EFFECT OF CO-SOLVENTS

Water is often a poor solvent for many pharmaceutical ingredients, but water is miscible with other solvents that may provide good solubility for these substances (e.g., ethanol, propylene glycol, polyethylene glycol, etc.). According to the log-linear model (*2*), the log *S* of the solute can generally be linearly interpolated between two miscible co-solvents. This relationship is illustrated in *Figure 2*. When this solubility plot is switched to a linear scale, it becomes evident that even low concentrations of the poor solvent (typically water) in the co-solvent mixture can dramatically reduce the solubility for the solute. For this reason, solutions containing co-solvents are particularly prone to precipitation when diluted due to the significant change in solubility. [NOTE—This simple model, as depicted in *Figure 2*, assumes that maximum solubility occurs at 100% of the good solvent and this may not be the case for all co-solvent systems.]

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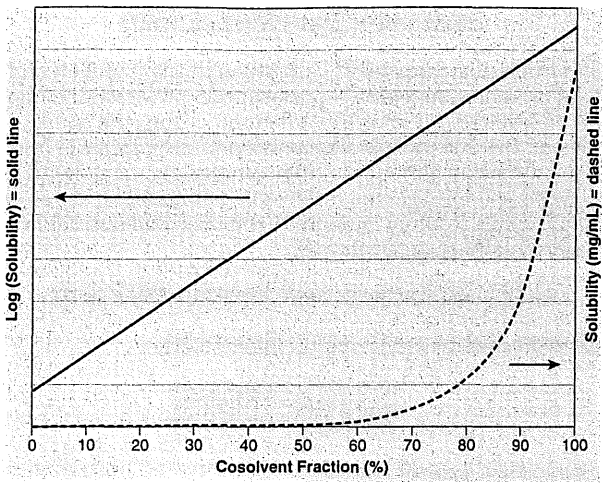

Figure 2. Illustration of the log-linear model of Yalkowsky and co-workers (*2*). The log of solubility appears to be linear as a function of the co-solvent fraction. When plotted on a linear scale, it is apparent that the solubility drops exponentially as the poor solvent is added to the better solvent.

EFFECT OF SURFACTANTS

Surfactants are amphiphiles, which are characterized by polar and nonpolar regions. When placed in water, a surfactant prefers to reside at the air-water interface and orients its polar region in water and its nonpolar region to the less polar interface (air). When the air-water interface is saturated with adsorbed surfactant, additional surfactant molecules aggregate into a spherical micelle with a polar surface and a nonpolar core. This point when micelles form is known as the critical micelle concentration (CMC). Above the CMC, the number of micelles in a solution increases linearly as the concentration of surfactant increases. If a pharmaceutical material is able to partition into the micelle, its solubility will increase linearly as the number of micelles increases (see *Figure 3*). The CMC for surfactants is dependent on several factors including temperature, ionic strength, and pH. As an example, the CMC for sodium lauryl sulfate is 6 mM, and the CMC for polysorbate 80 is 0.012 mM, in pure water at 25°. The solubilization of a molecule by a surfactant can be evaluated based on two descriptors: the molar solubilization capacity, and the micelle-water partition coefficient. The micelle-water partition coefficient is the ratio of drug concentration in the micelle to the drug concentration in water for a particular surfactant concentration (*7*).

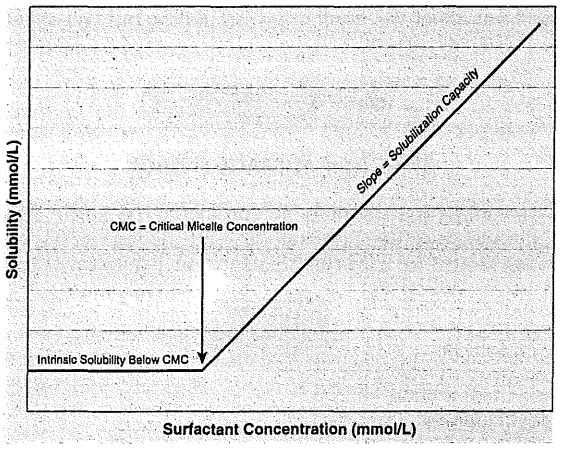

Figure 3. Solubility enhancement by surfactants. Solubilization requires the formulation of micelles. Below the CMC, added surfactant results in monomers of surfactant dissolved in the solvent and no solubility enhancement occurs. Above the CMC, the solubility increases linearly. The slope of this linear increase indicates the solubilization capacity of the micelles.

As illustrated in *Figure 3*, the solubility in the presence of surfactants is the additive combination of the amount dissolved in the aqueous phase plus the amount solubilized by the micelles. The micelles will be larger than the solute and will diffuse more slowly than the solute. Drug delivery in the presence of micelles will be due to the absorption of the free drug in solution as well as drug delivery by micelle-mediated transport (*5*,*6*). Therefore, solubilization by surfactants may not result in an enhancement in drug delivery that is directly proportional to the increase in aqueous solubility (*5*,*6*).

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EFFECT OF COMPLEXING AGENTS

Complexing agents may form intermolecular complexes with low-solubility materials and enhance the solubility. Aqueous solubility of nonpolar molecules in the presence of complexing agents is improved as the nonpolar molecules and the nonpolar region of the complexing agent are sequestered out of water. When this occurs, the aqueous solution can accommodate more of the nonpolar molecules. Regardless of the ratio of ligand to solute in these complexes (e.g., 1:1, 2:1, 3:1, etc.), the solubility enhancement is expected to increase as the concentration of the complexing agent increases (*8*). This is very similar to solubilization by surfactants except there will not be a minimum concentration of the complexing agent required. Complexes with high stability constants may bind solutes strongly enough to enhance aqueous stability. Cyclodextrins are often used to enhance solubility by forming complexes with drug substances.

EFFECT OF SURFACE AREA (DISSOLUTION RATE)

The Noyes–Whitney equation (as expressed by Nernst and Brunner) states:

$$\text{Rate} = \frac{\partial C}{\partial t} = \frac{DA(C_S - C)}{h}$$

C = concentration of the solute in the solvent at time, t
D = diffusivity of the solute
A = surface area of the solute particles
C_S = saturated solubility of the solute
h = thickness of the diffusion layer

In unstirred solutions, the diffusion layer thickness, h, may be large and is primarily affected by the diffusivity of the solute; however, good mixing may significantly reduce the diffusion layer thickness. For small particles in well-mixed solutions, the diffusion layer thickness, h, was found (*9*) to be proportional to the square root of the particle radius. This equation indicates that the smaller particles will have greater surface area and will dissolve more quickly. In order to reach the equilibrium solubility as quickly as possible, the surface area should be kept as high as possible (i.e., smaller particles) and the diffusion layer thickness kept as small as possible (i.e., good mixing) (*9*).

For spherical particles, the surface area, A, can be expressed as a function of the total mass, M:

$$\text{For spherical particles, } A = \frac{6M}{\rho d}$$

ρ = density
d = particle diameter

The dissolution rate of a material will not affect the equilibrium solubility, but will affect how quickly this equilibrium is achieved.

$$\text{Rate} = \frac{\partial C}{\partial t} = \frac{6DM}{\rho dh}(C_S - C)$$

EFFECT OF SURFACE ENERGY

The surface energy of the particle may affect the solubility. According to the Kelvin equation, smaller particles have higher solubility than larger particles due to the effect of the surface energy on the total Gibbs free energy of the system. Typically, this effect on solubility only becomes significant for particles smaller than 1 micron. The Kelvin equation quantifies this as:

$$\ln\frac{S}{S_0} = \frac{4\gamma V_m}{RTd}$$

S = apparent solubility
S_0 = solubility of an infinitely large particle
γ = surface energy of the solute
V_m = molecular volume of the solute
R = gas constant
T = temperature
d = diameter of the particle

The solubility difference between smaller and larger particles leads to so-called Ostwald ripening in polydisperse suspensions. The small particles dissolve and result in a solution that is supersaturated relative to the solubility of the larger particles. This leads to recrystallization on the surface of the larger particles. The larger particles grow in size while the smaller particles dissolve resulting in an increase in the mean particle size of the suspension (*10*).

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EXPERIMENTAL METHODS

Methods for Determination of Equilibrium Solubility

SATURATION SHAKE-FLASK METHOD

The shake-flask method is based on the phase solubility technique that was developed 40 years ago and is still considered by most to be the most reliable and widely used method for solubility measurement today (*11–18*). The shake-flask method should be used when equilibrium solubility needs to be determined. Other methods may be used to evaluate apparent solubility, but are not considered suitable for evaluation of true equilibrium solubility.

The solubility medium selected for solubility measurements should be selected to be relevant to the application with efforts made to control the surfactant type and concentration, the ionic strength of the buffer, and the types of counter-ions present in the buffer. When the results are intended to predict absorption or bioavailability, it is recommended that one of the biorelevant media solutions be used (see *Solubility Measurements in Biorelevant Media*). When the results are intended to support the development of dissolution tests, it is recommended that the dissolution medium be used. For research purposes, when the pH dependence of the compound is being evaluated, a buffer that allows control of ionic strength and counter-ion types over a wide pH range is recommended (e.g., Britton–Robinson or Sörensen buffers). For solubility measurements that will be used for BCS classification, USP-recommended buffers should be used (*19*).

Sample preparation: The test substance is typically prepared by adding an excess of solid to the solubility medium, which is in a stoppered flask or vial. The amount of medium in the flask or vial does not need to be measured accurately. It is recommended that the solid be added to the solubility medium at about 1–2 mg/mL in excess of the estimated solubility. (For low-solubility compounds, a concentration of 1–2 mg/mL may be sufficient.) The surface area of the solid may be increased by grinding (e.g., in a mortar and pestle) of the sample prior to addition to the medium or by sonication of the sample after addition to the medium. [**CAUTION**—It is advised to use caution when employing high-energy methods to increase the surface area because it may alter the solid form of the solute.] It is recommended that sample preparation be performed in triplicate to provide at least 3 solubility results for each test condition.

Equilibration of solution: To facilitate dissolution of the solid, the suspension should be actively mixed or agitated. As a good initial time of incubation, 24 h is recommended; however, the suitability of the selected equilibration time must be verified. The temperature of the suspension should be well controlled during this dissolution phase (±0.5°). Following the dissolution phase, it is recommended that the excess solid be allowed to sediment completely. Sedimentation and decantation is recommended as the safest method for separation of the solid from the saturated solution. For non-clarifying colloid solutions, centrifugation can be used. Sampling of the supernatant should avoid incorporating any undissolved solid, as this will significantly affect the solubility result. The transfer pipet needs to be pretreated with sample solution before use, so that surface adsorption is not altering the transferred solution. If filtration cannot be avoided, then it is essential that the proper filter type is selected. For polar, ionized species, hydrophobic type filters (nylon) are recommended; while for unionized species the hydrophilic type filters [e.g., polyvinylidene difluoride (PVDF) or polyethersulfone (PES)] are recommended. The filtration should be done after sedimentation, and not directly after agitation. Presaturation of the filter is necessary (i.e., the initial portions of filtrate should be discarded). The temperature of the suspension during the sedimentation and centrifugation steps must also be well controlled (±0.5°) and be equivalent to the temperature at which solubilization occurred.

Saturation (equilibrium) has been reached when multiple samples, assayed after different equilibration time periods, yield equivalent results (e.g., change by less than 5% over 24 h, or less than 0.2%/h). To confirm that the apparent solubility is the equilibrium solubility, it is recommended that the same suspension be re-equilibrated via the same procedure (e.g., mix for an additional 24 h).

Analysis of solution: The requirements for the analytical method used to quantitate the concentration of the solute and the level of analytical validation required should be commensurate with the intended use of the solubility data. In general, the method should be linear and specific. The supernatant solution may need to be diluted before analysis to be within the linearity of the analytical method and to avoid possible precipitation. The solution may be analyzed by UV-Vis spectrometry or by liquid chromatography methods to determine the soluble concentration. The advantage of HPLC is that it can detect instability by resolving drug-related impurities (*13,20*).

It is recommended that the excess solid in the suspension be analyzed at the end of the solubility measurement to verify that the solid form has not changed. In cases where the solid form has changed, it is likely that the new solid form has a lower solubility than the initial solid form and that the observed solubility is due to the new lower-solubility form; however, this should be evaluated on a case-by-case basis. Powder X-ray diffraction (PXRD), Raman or near infrared (NIR) spectrometry, or evaluation of the melting point by differential scanning calorimetry (DSC) are examples of techniques that can be used to evaluate the solid form. Solutes that are unstable (either chemically or physically) during the equilibration time are not suitable for equilibrium solubility measurements by the shake-flask method. For example, amorphous drugs that will convert to lower solubility salts or polymorphs should be analyzed using one of the apparent solubility methods.

Reporting of solubility results: If a non-standard composition for the media is used in the solubility determination, the details of the composition should be reported. The ionic strength of the media used in the solubility determination should be calculated and reported with the solubility result. The pH of the supernatant solution should be recorded (at the temperature of the solubility measurement) when the sample is withdrawn for analysis. When using well-defined, standard media, it is recommended that the pH of the media not be adjusted to compensate for alteration of the pH by the dissolving species; rather, the solubility value should be reported at the pH value and temperature observed at the end of the equilibration step (*12,18*). If the pH of the media is significantly affected by the dissolving species and solubility at a particular pH is desirable, it is recommended to perform an additional solubility measurement in a higher buffer-capacity medium. Report the mean temperature and the precision of temperature control during the equilibration.

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The precision of the reported equilibrium solubility should reflect the level of agreement between the measurements rather than the precision of the solubility analysis. Standard deviations in the measured solubility (based on averaging 3 or more independent samples) should be included.

METHODS FOR DETERMINATION OF APPARENT SOLUBILITY

Intrinsic Determination (Rotating Disk)

The measurement of intrinsic dissolution is described more fully in *Apparent Intrinsic Dissolution—Dissolution Testing Procedures for Rotating Disk and Stationary Disk* ⟨1087⟩. This measurement technique may also be used to assess the solubility.

To apply this method to the measurement of solubility, the dissolution experiment must be continued to the point that the rate of dissolution is insignificant (e.g., less than 5%/24 h or less than 0.2%/h).

All the requirements discussed for the shake flask method in *Analysis of solution* and *Reporting of solubility results* will also apply to measurements using the intrinsic dissolution apparatus.

Potentiometric Titration

The potentiometric acid–base titration for solubility measurements is based on a characteristic shift in the middle of the titration curve that is caused by precipitation (*21*). For the titration, accurate volumes of a standardized acid or base are added to a solution containing an ionizable substance and a salt, for example, 0.15 M potassium chloride (KCl), which is included to increase the accuracy of the measurements. Sparging (a technique that involves bubbling a chemically inert gas such as nitrogen, argon, or helium through a liquid) with argon prevents carbon dioxide (CO_2) from the atmosphere from influencing the pH value. A glass electrode is used to monitor the pH value continuously. The potentiometric titration curve is obtained by plotting the pH value against the consumed volume of acid/base (*21*).

Turbidimetry

Turbidimetry involves the dissolution of a compound in an organic solvent, for example, dimethyl sulfoxide (DMSO). The resulting solution is added to a buffer solution in intervals adequate to characterize changes in turbidity. Further aliquots of the solution are added after the first detection of turbidity by light scattering. Subsequently, the volume added can be plotted against the turbidity. The solubility is then estimated by back-extrapolation to the point where precipitation began. This method can be used to measure as many as 50–300 samples per day. When using solvents such as DMSO, drawbacks include the increase in solubility of the drug substance for the short duration of the experiment, which leads to a kinetic rather than thermodynamic solubility, the formation of a supersaturated solution, and the undefined crystalline form of the precipitated solid (unless it is removed from the suspension and characterized).

Physical Assessment of Solubility

For compounds with extremely high solubility, as well as for biologics and other molecules that lack chromophores or are not easy to quantitate in solution, a physical assessment of solubility may be used to assess apparent solubility. In this case, the principle of measurement is the loss of solid material to the solution phase. Equilibrium may be assessed by both the stability of the weight loss as well as the stability of the change in the physical properties of the resulting solution (e.g., refractive index, density, osmolality, etc.). Because the physical assessment of solubility does not involve a specific or stability-indicating assay, it is recommended that some attempt be made to verify the stability and purity of the solute. Also, evaporation of the solvent should be carefully monitored and controlled during the solubility measurement performed by this method.

SOLUBILITY MEASUREMENTS IN BIORELEVANT MEDIA (*22–26*)

The use of simple aqueous buffers to evaluate the aqueous solubility of a drug substance as a function of pH may underestimate the bioavailability (*22,23*). The media recipes presented here are examples that may be used to evaluate the solubility in simulated human, canine, and bovine (ruminant) fluids to make improved estimates of bioavailability.

The temperature of the solubility medium should be controlled at ±0.5° during biorelevant solubility measurements. Biorelevant solubility measurements should follow the shake-flask method, including solubility measurements at multiple time points to confirm that equilibrium has been achieved. The salt form of a drug added to the solubility medium may significantly affect the composition of the medium (i.e., ionic strength, pH, etc.). Therefore, the solubility of a salt and a free base of the same drug should not be assumed to be equivalent unless proven through independent measurements starting with the different crystalline solids.

Human Fasted-State Simulated Gastric Fluid (FaSSGF) (*24*)

The pH is 1.6 at 37°. See *Table 1* for the media composition.

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Table 1

Ingredient	Concentration (mM)	Concentration (g/L)
Hydrochloric acid	~31.3 (q.s. to pH 1.6)	~1.14 (q.s. to pH 1.6)
Sodium chloride	34.2	2.00
Sodium taurocholate	0.08	0.047
Lecithin	0.02	0.015
Pepsin	—	0.1

Human Fed-State Simulated Gastric Fluid (FeSSGF) *(24)*

The pH is 5 at 37°. See *Table 2* for the media composition.

Table 2

Ingredient	Concentration (mM)	Concentration (g/L)
Hydrochloric acid	(q.s. to pH 5)	(q.s. to pH 5)
Sodium hydroxide	(q.s. to pH 5)	(q.s. to pH 5)
Sodium chloride	237.0	13.85
Sodium acetate	29.75	2.441
Acetic acid	17.12	1.028
Milk, whole	1:1	1:1

Prepare buffer and then mix 1:1 with milk. Adjust to a pH of 5, if necessary.

Human Fasted-State Simulated Intestinal Fluid (FaSSIF-V2) *(24)*

The pH is 6.5 at 37°. See *Table 3* for the media composition.

Table 3

Ingredient	Concentration (mM)	Concentration (g/L)
Maleic acid	19.12	2.219
Sodium hydroxide	34.8	1.392
Sodium chloride	68.62	4.010
Sodium taurocholate	3.0	1.766
Lecithin	0.2	0.165

Human Fed-State Simulated Intestinal Fluid (FeSSIF-V2) *(24)*

The pH is 5.8 at 37°. See *Table 4* for the media composition.

Table 4

Ingredient	Concentration (mM)	Concentration (g/L)
Maleic acid	55.02	6.338
Sodium hydroxide	81.65	3.226
Sodium chloride	125.5	7.460
Sodium taurocholate	10	5.89
Lecithin	2.0	1.65
Glyceryl monostearate	5.0	1.79
Sodium oleate	0.8	0.24

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Human Simulated Colonic Fluid—Proximal Colon (SCoF2) (*25*)

The pH is 5.8 at 37°. See *Table 5* for the media composition.

Table 5

Ingredient	Concentration (mM)	Concentration (g/L)
Sodium hydroxide	~159 (q.s. to pH 5.8)	~5.4 (q.s. to pH 5.8)
Acetic acid, glacial	170	10.2

Human Simulated Colonic Fluid—Distal Colon (SCoF1) (*25*)

The pH is 7.0 at 37°. See *Table 6* for the media composition.

Table 6

Ingredient	Concentration (g/L)
Potassium chloride	0.2
Sodium chloride	8
Potassium phosphate, monobasic	0.24
Sodium phosphate, dibasic	1.44

Canine Fasted-State Simulated Gastric Fluid (FaSSGFc pH 1.2–2.5) (*26*)

Canine gastric pH can vary substantially. Due to interstudy variations in the canine gastric pH estimates, solubility should be evaluated over a range of 1.2–6.5. The pH of this fluid is adjusted by altering the amount of hydrochloric acid so that pH values in the range of 1.2–2.5 may be achieved. The pH is 1.2–2.5 at 37°. See *Table 7* for the media composition.

Table 7

Ingredient	Concentration (mM)	Concentration (mg/mL)
Hydrochloric acid	~3.6–82 (q.s. to pH 1.2–2.5)	~0.13–3 (q.s. to pH 1.2–2.5)
Sodium chloride	14.5	0.847
Sodium taurocholate	0.10	0.055
Sodium taurodeoxycholate	0.10	0.054
Lecithin	0.025	0.019
Lysolecithin	0.025	0.012
Sodium oleate	0.025	0.0076

Canine Fasted-State Simulated Gastric Fluid (FaSSGFc pH 2.5–6.5) (*26*)

Canine gastric pH can vary substantially. Due to interstudy variations in the canine gastric pH estimates, solubility should be evaluated over a range of 1.2–6.5. The pH of this fluid is adjusted by altering the amount of sodium hydroxide so that pH values in the range of 2.5–6.5 may be achieved. The pH is 2.5–6.5 at 37°. See *Table 8* for the media composition.

Table 8

Ingredient	Concentration (mM)	Concentration (g/L)
Maleic acid	21.68	2.516
Sodium hydroxide	~14.5–40 (q.s. to pH 2.5–6.5)	~0.58–1.6 (q.s. to pH 2.5–6.5)
Sodium chloride	18.81	1.099
Sodium taurocholate	0.10	0.055
Sodium taurodeoxycholate	0.10	0.054
Lecithin	0.025	0.019
Lysolecithin	0.025	0.012

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Table 8 *(continued)*

Ingredient	Concentration (mM)	Concentration (g/L)
Sodium oleate	0.025	0.0076

Canine Fasted-State Simulated Intestinal Fluid (FaSSIFc) *(26)*

The pH is 7.5 at 37°. See *Table 9* for the media composition.

Table 9

Ingredient	Concentration (mM)	Concentration (mg/mL)
Sodium dihydrogen phosphate, monohydrate	28.65	3.953
Sodium hydroxide	21.66	0.866
Sodium chloride	59.63	3.485
Sodium taurocholate	5.0	2.8
Sodium taurodeoxycholate	5.0	2.7
Lecithin	1.25	0.96
Lysolecithin	1.25	0.62
Sodium oleate	1.25	0.38

Bovine Simulated Ruminal Fluids *(27–29)*

The media defined here are suitable to represent bovine as well as other ruminant species. The normal pH of a healthy reticulo-rumen is in the 5.5–6.8 range. High-grain diets typically result in a lower ruminal pH (~5.5), whereas high-forage diets result in a higher ruminal pH (~6.8).

In the abomasum (true stomach), the pH is about 2–3 and is similar to conditions observed in monogastrics and humans. To represent the abomasum, one may use 0.01 M hydrochloric acid (pH 2), 0.0033 M hydrochloric acid (pH 2.5), or 0.001 M hydrochloric acid (pH 3). Intestinal pH in ruminants is similar to that observed in monogastrics and humans. The pH at the pylorus is about 3.0 and increases to about 7.5 in the ileum. To represent the bovine intestinal fluids, one may use one of the simulated intestinal fluids defined for human or canine above.

The temperature is set for 39°. [NOTE—The pH of media should be confirmed at 39°.] See *Table 10* for the media composition.

Table 10

Ingredient	High-Grain Diet Simulated Rumen	High-Forage Diet Simulated Rumen
Tryptone	2.5 g/L	2.5 g/L
Sodium bicarbonate	4.0 g/L (47.6 mM)	8.75 g/L (104 mM)
Potassium phosphate monobasic anhydrous	1.55 g/L (11.4 mM)	1.55 g/L (11.4 mM)
Sodium phosphate dibasic anhydrous	1.425 g/L (10.0 mM)	1.425 g/L (10.0 mM)
Ammonium bicarbonate	1.0 g/L (12.6 mM)	1.0 g/L (12.6 mM)
Magnesium sulfate heptahydrate	0.15 g/L (0.6 mM)	0.15 g/L (0.6 mM)
Acetic acid	2.40 g/L (40 mM)	1.42 g/L (24 mM)
Propionic acid	2.59 g/L (35 mM)	—
Butyric acid	1.32 g/L (15 mM)	—
Sodium acetate trihydrate	—	6.26 g/L (46 mM)
Sodium propionate	—	1.44 g/L (15 mM)
Sodium butyrate	—	1.10 g/L (10 mM)
Micromineral stock solution (see *Table 11*)	125 µL	125 µL
pH	5.5	6.8
Surface tension	~50 dynes/cm²	~50 dynes/cm²
Ionic strength	0.102 mol/L	0.240 mol/L
Buffer capacity	47 mmol/L/pH	49 mmol/L/pH

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Table 11

Ingredient	Micromineral Stock Solution Concentration (g/100 mL)
Water	q.s. to 100 mL
Calcium chloride dihydrate	13.2
Manganese chloride tetrahydrate	10.0
Iron (III) chloride hexahydrate	8.0

GLOSSARY

[NOTE—The following definitions are provided to clarify the use of these terms in the context of this chapter. These definitions are not intended to supersede or contradict definitions found elsewhere in the *USP–NF*.]

Apparent solubility: The empirically determined solubility of a solute in a solvent system where insufficient time is allowed for the system to approach equilibrium or where equilibrium cannot be verified. The apparent solubility may be either higher or lower than the equilibrium solubility due to transient supersaturation or incomplete dissolution and insufficient time to reach equilibrium.

Aqueous solubility: Solubility in a medium that is primarily comprised of water but may also contain solubilization enhancement from co-solvents, surfactants, complexing agents, pH, or other co-solutes. The term "aqueous solubility" is very general and should not be confused with the water solubility. The aqueous solubility is significantly affected by the composition of the aqueous medium.

Dissolution: The non-equilibrium process of approaching the solubility limit at thermodynamic equilibrium (i.e., the solute and solvent forming a uniformly mixed solution). The dissolution rate will affect the time required to reach equilibrium and may affect the apparent solubility, but will not affect the final equilibrium solubility.

Equilibrium solubility: The concentration limit, at thermodynamic equilibrium, that a solute can dissolve into a saturated solution when excess solid is present. Equilibrium can be defined as sufficiently converged when it no longer changes significantly during a certain time frame.

Intrinsic solubility: The solubility of the uncharged (neutral) moiety. Intrinsic solubility can only be accurately measured in pH ranges where the distribution of species is dominated by the uncharged molecule. For some compounds, it may be impossible to directly measure the intrinsic solubility and it must be determined by fitting the solubility data as a function of pH.

Solubility: The extent to which a solute may be uniformly dissolved into a solvent. This may be referred to as equilibrium (saturated) solubility to differentiate it from apparent solubility. Solubility may be stated in units of concentration such as molality, mole fraction, mole ratio, weight/volume, and weight/weight.

Water solubility: Solubility in pure water. Measurements of solubility in pure water are problematic due to poor control of pH and ionic strength.

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⟨1237⟩ VIROLOGY TEST METHODS

INTRODUCTION

This chapter describes virology test methods applicable to the development of biological product drugs, such as recombinant proteins, subunit vaccines, therapeutic monoclonal antibodies, and growth hormones. Several topics are excluded from the scope of this chapter:

- Blood- and plasma-derived products as well as whole blood and plasma products used directly in transplantation or infusion. However, the basic principles, strategies, and testing methods for ensuring virus-free products are applicable.
- Methodologies for the safety testing of live viral vaccines.
- Specific methods for viral clearance studies, which are described in the *USP* general information chapter *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

Virology test methods have historically been employed in the clinical settings of disease diagnosis, intervention, and containment; but the development of biological (biologics and biotechnology-derived) products and therapies for human or animal use has created the need for sensitive viral detection assays for use in the GMP production and testing of biological products. This need is not limited to the production of viral vaccines, but also applies to the development and manufacture of recombinant proteins, cell and gene therapies, and other products.

Sensitive virology test methods for quality control of biological products are necessary for several reasons. The production of biological products often requires a variety of raw materials and processing reagents of animal origin that have varying potential for introducing viral contaminants. The production of biological products may allow the replication of adventitious agents during processing, and therefore these materials must be prescreened to avoid the opportunity for contamination of the product. Another point to consider regarding screening these materials is that the product may not be compatible with processing methods used to eliminate or inactivate these adventitious agents. Because of the nature of the biological products, the production process needs to include appropriate testing regimens that monitor the possible introduction of adventitious agents and/or viral agents into the systems used. For these reasons, sensitive viral detection methods are required not only for the release testing of biological drug products, but also during the intermediate stages of processing, process development, and routine manufacture. Important stages for consideration include the development of cell substrates and banks, raw materials of animal origin, process intermediates, and critical excipients when derived from animal tissues. This strategy should be augmented with viral clearance and inactivation studies whenever possible.

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For products intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy), the cell- and animal-based infectivity methods discussed in this chapter may be useful only following neutralization of the specific viral entity contained in the product for any product that is intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy). Alternatively, selection of appropriate indicator cell lines or animal models in which the specific viral entity is known not to replicate can be considered. It should also be expected that assay systems based on detection of viral particles or viral components will indicate the presence of the viral entity itself in such products, but may not indicate the viability of the virus. The remainder of the chapter is divided into three sections discussing assays for the three topics: (1) *Detection of Viable Viruses,* (2) *Detection of Viral Components,* and (3) *Detection of Antibodies to Viral Antigens.* The chapter covers the classic virology methods that are still routinely used, as well as modern molecular and immunological approaches. The methods described in these sections may possess different sensitivities to diverse viruses; they are therefore intended to complement each other to provide a science-based foundation for the detection of adventitious viruses. Multiple methods may be used in complementary fashion to improve the pathogen safety margin of a product. Identification of viruses detected in cell-based assays on the basis of cytopathic effects often depends on the use of molecular and immunological analyses; these analyses are therefore relevant both to viral detection and to subsequent viral identification. The chapter provides an overview of the detection and analysis of the most important groups of viruses as well as the most commonly used techniques. Tests specific to individual vaccines or biological products are excluded, because they are expected to be included in monographs for such products.

Methods that are well established with little variation in practice are described in more detail, whereas methods that are more flexible are described in general terms, both in the performance of the tests and in considerations for acceptance. Relevant regulatory references are given in the *Appendix.* Relevant USP general chapters should be consulted with regard to bioassay design, data analysis, interpretation, and assay validation.

DETECTION OF VIABLE VIRUSES

Infectious virus particles contaminating biologics and biotechnology-derived products are of great safety concern, because they have the potential for causing serious, possibly life-threatening, infections in the patients treated. This is particularly true if the patients are immunocompromised. Although complete assurance of viral safety for finished biological products can never be realized, a significant safety margin can be established through viral detection methods applied to unprocessed bulk and raw materials before purification in combination with purification processes that demonstrate the ability to inactivate or remove potential viral contaminants present at levels too low to detect. (See *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ for information on viral clearance and inactivation methods.) For cell and gene therapy products that lack extensive purification steps, final products may be directly tested for the presence of relevant contaminating viruses. This section describes two broad systems for the detection of infectious virus: cell culture–based infectivity assays and in vivo infectivity assays. These systems may possess complementary sensitivities for viruses, and as a result, both methods may be used as limit tests for cell bank and raw material characterization and for lot release testing of biologics and biotechnology-derived products. Considerations for optimizing sample preparation for these tests are discussed, followed by a description of the more commonly employed detection assays. Finally, to ensure the reliability of experimental results, quality control issues in general and detection limit estimation in particular are discussed.

Sample Selection of and Preparation for Cell- and Animal-Based Virus Detection Assays

The requirements for selection, preparation, and storage of test samples for viral detection methods (cell- and animal-based) are dictated by the lability of the viruses being detected. The ability of a virus to remain infectious in the absence of a host cell is highly variable. Virus infectivity also may differ in sensitivity to repeated freezing and thawing cycles.

Sample preparation typically involves storage of test samples at low temperatures (ideally –60° or below) as soon as practicable upon collection. When intended for use in a viral screening assay, aliquots of samples should be prepared to avoid multiple freezing and thawing. Samples intended for viral infectivity assays are typically shipped with sufficient dry ice to last several days more than the expected time required for transit. When received at the testing laboratory, the sample should be examined to verify that it is still frozen, and appropriate documentation should be completed. For any storage or hold condition, the impact of the condition on viral viability should be empirically assessed and sufficient cold chain management ensured.

Typical sample types for viral detection assays are described below.

CELL LYSATES

Test samples derived from cell substrates (master and working cell banks, end-of-production cell samples) are prepared in a manner that allows sampling of both the cells (for cell-associated viruses) and the conditioned medium (for virus shed into the medium). To achieve this, a culture of the cells is sampled. A cell suspension of ~10^7 cells per mL in conditioned medium is prepared and frozen (ideally at –60° or below). Because this medium does not contain cryopreservative, the majority of the cells will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation will remove larger cellular debris and yield a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. A similar sample is prepared for in vivo viral adventitious agent testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

BIOTECHNOLOGY BULK HARVEST (UNPROCESSED BULK HARVEST) SAMPLES

Routine lot testing of bulk harvest samples is mandatory for most types of biologics (see *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩). The sampling must be done at the unprocessed bulk harvest stage, because downstream purification processes may remove or inactivate any viruses that might contaminate the starting

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materials. The harvest sample from the bioreactor should be collected and stored without further manipulation, as soon as practicable, at –60° or below. To prevent multiple freeze and thaw cycles, individual aliquots should be prepared for each individual assay to be performed. Additional aliquots should be retained in case repeat testing is required. Depending on the nature of the manufacturing process, the bulk harvest samples may contain varying quantities of the production substrate cells. Because the bulk harvest does not contain cryopreservatives, the majority of the cells present will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation to clarify the sample will result in a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. There may be instances where the test sample is cytotoxic to the detector cells of the cell-based assays and procedural modifications may be required to deal with this. A similar sample is prepared for in vivo viral safety testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

RAW MATERIALS OF ANIMAL ORIGIN

Ingredients of animal origin used in the manufacture of biological products for human or veterinary use must be tested for species-specific viruses of concern as described in 9 CFR 113.53 (see also the *USP* general information chapter *Bovine Serum* ⟨1024⟩, being prepared for future publication). The raw materials may be stored under a variety of conditions, as appropriate to the raw material. Sample preparation and method of application to the test system depend on the nature of the sample. The possibility that animal-derived raw materials may contain bacterial or fungal contaminants should be considered. In some cases, it may be necessary to treat the samples with antibiotics or to filter the samples (0.22 or 0.45 micron pore size) prior to inoculation in order to prevent bacterial or fungal outgrowth in the test system. Animal sera are typically received frozen and are thawed and incorporated into the growth medium at an appropriate concentration (typically 15%, v/v) as a means of exposing the detector cells. Powdered trypsin (not less than 5 grams, as per 9 CFR 113.53) is suspended in a suitable diluent, such as phosphate-buffered saline, and is then subjected to high-speed centrifugation to pellet any virions that may be present. The concentrated pellet is resuspended in phosphate-buffered saline, and the resulting material is used to inoculate appropriate detector cells. Medium additives, such as bovine thrombin, may be incorporated into the growth medium at a predetermined multiple of the nominal concentration to be used in the manufacturing process. The resulting growth medium containing the additives is then used as a means of exposing the detector cells to the test material. The exact multiple to be used in such testing may be limited by such factors as solubility in growth medium or cytotoxicity to the detector cells. These factors should be assessed in advance of testing. The principle of using higher concentrations in the detection method than during processing should be followed, within the bounds of indicator cell toxicity, as a means to increase sensitivity to detection.

WHOLE CELLS

Intact viable cells are used as the test sample in certain viral detection assays. Because the test cells may attach and proliferate in the culture vessel along with the detector cells, assays using this type of sample are referred to as cocultivation assays. The requirements for the specific assay may vary in relative proportions of detector and test cells, viability of the test cells, or the confluency of test cells at the time of collection.

Cell Culture-Based Viral Detection Methods

To ensure the absence of adventitious viral agents, cell culture–based viral detection assays are used for a variety of purposes, including but not limited to clinical diagnostic procedures; evaluation of raw materials and cell substrates; assessments of the viral identity, the purity, and the potency of virus seed stocks; and lot release testing of unprocessed bulk harvests during biologics production. An important distinction between cell-based assays and direct detection assays (see the section *Detection of Viral Components*) is that the former will detect only replicating virus, whereas the latter will detect viral antigens, viral genomic material, and the like, which may or may not be indicative of the presence of replicating virus. Similarly, detection of circulating antibodies directed against viral antigens (discussed in the section *Detection of Antibodies to Viral Antigens*), may be indicative of either a current or a past infection of an animal and does not necessarily indicate that the animal is currently harboring an infection.

Infectious viruses detected in cells or in cell-derived materials fall into two broad categories, based on the expectations of the analyst. Endogenous viruses are those normally detected in the cells as a result of the integration of the viral genomic material into the host cell DNA. Exogenous viruses are those not normally present in the cells but found as a result of a viral infection of the cells.

The underlying assumption for all cell-based viral detection methods is the ability of viruses to replicate in an appropriate host cell. Viruses lack the cellular machinery required for producing their own genomic material and structural proteins, and they must therefore enter and subordinate a host cell for this purpose. Cell-based viral infectivity assays use indicator (detector) cells that serve as host cells for viable virions present in test samples.

Cell-based infectivity assays may be placed in three broad categories on the basis of types of viruses to be detected: (1) retroviral assays, (2) virus-specific assays, and (3) viral screening assays. The types of endpoints used to detect the viruses may differ by category. Although screening assays are typically not optimized for single viral entities, the virus-specific assays and titration assays, as well as some of the retroviral assays, may be optimized to some extent for specific viruses. Accurate titration of stock viruses that are used as positive controls or are used to determine the detection limit of an assay is critical.

The regulatory guidance underlying the various viral safety tests depends on the nature of the samples to be evaluated, and analysts are referred for more detail to documentation relevant to their own regulatory environments.

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General Requirements for Cell Culture–Based Assays

DETECTOR CELLS AND THE CONCEPT OF VIRAL HOST RANGE

The range of viruses detectable using a cell-based infectivity assay depends on a number of factors, including the type of host cell(s) used as the indicator (detector) cultures and the detection endpoints used in the assay. Viruses differ in their abilities to infect specific host cell types. Most viruses exhibit at least some degree of host cell tropism (i.e., ability to infect a specific species or tissue type). This attribute is typically due to a requirement for interaction of a virion with a specific cell membrane receptor during the process of infection of the host cell. A cell susceptible to infection and capable of production of progeny by a given virus is referred to as *permissive* for that virus; cells not supporting viral proliferation are referred to as *nonpermissive,* or *restricted,* for that virus. As a consequence of the differences in host cell tropism, assays intended to screen for a wide range of viruses must include multiple detector cell types. For the same reasons, design of a cell-based infectivity assay for a specific virus must include a detector cell known to be permissive for that virus.

VIRUS SUSCEPTIBILITY OF COMMON CELL LINES

For most endpoint assays used to determine whether a host cell is infected with a virus, a monolayer culture is preferable to a semiadherent or suspension culture. For instance, cytopathic effect and hemadsorption are visualized microscopically. Cells that are not adherent have little morphology to evaluate, and hemadsorption cannot be properly evaluated in a suspension culture. For this reason, some regulatory documents pertaining to cell-based virus infectivity assays stipulate the use of monolayer detector cultures.

A list of commonly employed indicator cell lines and their application in viral screening assays is provided in *Table 1*. Regarding the viral tropism of these cells, "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993) and ICH's Q5A (R1) guidelines (for these references, see *Appendix*) require that human diploid cells such as MRC-5 and WI-38, which are permissive for a range of viruses of human concern, and monolayer cultures of the same species as that of the cell substrate used to produce the product are included in the viral screening test for biologics destined for use in humans.

Table 1. Indicator (Detector) Cell Lines Used for Adventitious Viral Screening Assays

Cell Line[a]	Origin	Endpoint(s)[b]	Target virus(es)
Cell lines with relatively broad viral tropism:			
BHK-21	Syrian hamster	CPE, HAd, HA	Insect-borne viruses (arboviruses)
Vero	African green monkey	CPE, HAd, HA	Viruses infectious to humans, primates
For processes involving human cell substrates:			
HeLa	Human	CPE, HAd, HA	Viruses infectious to humans
MRC-5	Human	CPE, HAd, HA	Viruses infectious to humans
For processes involving Chinese hamster cell substrates:			
CHO-KI	Chinese hamster	CPE, HAd, HA	Viruses infectious to Chinese hamsters
For processes involving mouse cell substrates:			
MEF	Mouse	CPE, HAd, HA	Viruses infectious to mouse cells
NIH/3T3	Mouse	CPE, HAd, HA	Viruses infectious to mouse cells
For processes involving bovine cell substrates or bovine raw materials:[c]			
MDBK	Bovine	CPE, HAd, HA	Bovine viruses
BT	Bovine	CPE, HAd, HA	Bovine viruses
EBTr	Bovine	CPE, HAd, HA	Bovine viruses

[a] Examples of cell lines used for viral screening assays are shown. MRC-5 and Vero, or cells with similar host ranges, are used in all assays. Depending on the cell substrate used to manufacture a biologic, additional cell lines are also used in the screening assay. In addition, a bovine cell might be included if bovine serum was used in the manufacturing process.

[b] CPE, cytopathic effect; HAd, hemadsorption; HA, hemagglutination (optional).

[c] Inclusion of a bovine cell in a virus screen should not be construed as a replacement for or alternative to a raw materials test. Raw materials testing is driven in the U.S. by 9 CFR 113.47 and 113.52, and cell lines used for this testing are described in *Table 2*.

A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in *Table 2*.

Table 2. Indicator (Detector) Cell Lines Used in Raw Material Testing

Cell Line[a]	Assay Type	Endpoint(s)[b]	Animal Origin of Raw Material
Vero	Isolation/detection	CPE, HAd, IFA	All sources
BT	Isolation/detection	CPE, HAd, IFA	Bovine; all sources (BVDV)[c]
EBTr	Isolation/detection	CPE, HAd, IFA	Bovine; all sources (BVDV)[c]
MDBK	Isolation/detection	CPE, HAd, IFA	Bovine; all sources (BVDV)[c]

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Table 2. Indicator (Detector) Cell Lines Used in Raw Material Testing *(continued)*

Cell Line[a]	Assay Type	Endpoint(s)[b]	Animal Origin of Raw Material
PT-1	Isolation/detection	CPE, HAd, IFA	Porcine
PK-1	Isolation/detection	CPE, HAd, IFA	Porcine
MDCK	Isolation/detection	CPE, HAd, IFA	Canine
GT	Isolation/detection	CPE, HAd, IFA	Caprine

[a] The requirement (9 CFR 113.47 and 113.52) for evaluating raw materials of animal origin is to use (1) Vero cells, (2) a bovine cell for detecting BVDV, and (3) a cell line of the same species of origin as the raw material for detecting viruses of concern from that species. Examples are given of some cell lines that are used in the industry.
[b] CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.
[c] As per 9 CFR 113.47, raw materials of any animal origin are to be tested for bovine viral diarrhea virus (BVDV).

There may be very specific requirements for detector cells for certain viruses. For instance, assays intended to detect infectious HIV use human peripheral blood lymphocytes and involve a p24 antigen capture enzyme immunoassay endpoint. A list of commonly employed indicator cell lines and their application in the detection of specific viruses is provided in *Table 3*.

Table 3. Indicator (Detector) Cell Lines Used for Detection of Specific Virus(es)

Cell Line[a]	Assay Type	Endpoint(s)[b]	Target Virus
324K	Isolation/detection	CPE, HAd, IFA	Murine minute virus
A9	Isolation/detection	CPE, HAd, IFA	Murine minute virus
BHK-21	Isolation/detection	CPE, HAd	Arboviruses[c]
MRC-5[d]	Isolation/detection	CPE	Human cytomegalovirus

[a] Examples of cell lines used for optimizing the detection of specific viruses or virus types are shown. In many cases, the assay methodologies must also be optimized for detection of the target viruses.
[b] CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.
[c] Insect-borne viruses as a group are referred to as arboviruses. This term has no taxonomic significance.
[d] Other human diploid cell lines such as WI-38 are also suitable. Assay duration must be 28 days at a minimum.

GROWTH REQUIREMENTS FOR DETECTOR CELLS

Viral proliferation within a permissive host cell may be dependent on the rate of host cell proliferation. This is especially true for viruses that display cell-cycle dependence for generation of viral progeny. For most detection assays, detector cultures are seeded at a density intended to achieve a cell monolayer in exponential growth. This corresponds to a cell confluency of 50% or less (optimal cell densities may depend on the assay type and the detector cell to be used) at the time of inoculation of the cultures with virus or test sample. For the same reasons, the assay design may include provision for detector cell subculture (the collection of cells from the original culture and seeding of a predetermined fraction of these into a new flask). Alternatively, a passage may be performed, consisting of collection of conditioned medium from the original culture and inoculation of this material onto a secondary detector cell culture that is in log-phase growth. The frequency of subculture/passage required in an assay is determined largely by the rate of growth of the detector cell. Incorporation of these steps into detection assay designs helps to ensure that the conditions remain optimal for amplification of viral progeny within the host cells.

NEED FOR DETECTOR CELL IDENTIFICATION AND BANKING

Detector cells used for cell-based viral detection assays are a critical reagent for ensuring viral safety, viral potency, viral identity, and viral clearance capacity in purification schemes. In many cases this testing is intended to support GMP processes; therefore, the detector cell banks may need to be prepared and qualified in much the same manner as other critical reagents. The details of the viral safety evaluation methods are described in *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩. Regardless of specific compliance requirements, periodic identification and qualification of the detector cell banks to be used subsequently for viral infectivity assays is good practice. The following should be regarded as minimal quality control testing for such detector cell banks: sterility, mycoplasma and viral screening, and cell identity by DNA fingerprinting, karyology, or isoenzyme analysis. In addition, specific assays for bovine and porcine viruses may be required if bovine or porcine raw materials were used in preparation of the banks. *Bovine Serum* ⟨1024⟩ and relevant serum-type specific ancillary materials monographs should be consulted when serum products are used in cell growth media.

Endpoints for Detection of Viral Infection

The various endpoints used to identify infection of a detector cell with a virus include the following:

- Visual observation of cytopathic effects
- Hemagglutination or hemadsorption of erythrocytes
- Immunofluorescent staining
- Cocultivation with other types of detector cells
- Quantitative polymerase chain reaction (qPCR) for direct detection of viral genomic sequences
- Electron microscopic analysis of viral pellets or fixed cells for visual observation of viral particles

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- Biochemical endpoints such as reverse transcriptase assays, which detect virus-specific enzymatic activity

These various endpoints are used in a complementary fashion, because a given virus may not cause a positive response in each endpoint. For instance, some viruses can grow to high titers without producing visible cytopathic effects and so must be detected using other endpoints. Polymerase chain reaction (PCR) and electron microscopic analysis per se are not capable of distinguishing viable from nonviable viruses. However, when used in conjunction with cell culture growth kinetics, these approaches can be powerful orthogonal detection methods to demonstrate the increase of viral replication and therefore viable virus. The failure to observe viral particles in electron microscopic analysis of fixed cells should not be considered absolute proof of the absence of infectious virus in the cells. In a general sense, the same is true for each of the detection endpoints discussed above. Each endpoint has a detection limit below which a virus may be present but not detected.

VIRAL CYTOPATHIC EFFECTS

Visually observable manifestations of the infection of susceptible host cells with certain types of viruses are collectively referred to as viral cytopathic effects (CPE). Although CPE may be considered an indirect detection of viral infection, in the context of specific host cells they can have distinctive morphological manifestations. These may include the appearance of inclusion bodies, abnormal cell morphology, changes in culture confluency, cell death and cell lysis, and others. The nature of the CPE observed may depend on the host cell and the infecting virus. In addition, for a virus that normally causes CPE, there may exist variants that do not cause CPE. CPE can be differentiated from cytotoxic effect by the tendency of the former to exhibit progression irreversibly with time, whereas the latter may be reversible. In some cases structural proteins of viruses may cause cytotoxic effects similar to the cytopathic effects of the infectious virus. Differentiating the cytotoxic effect of such proteins from the cytopathic effect of infectious virus may require observation of the culture over time to determine whether the effect progresses or the cells appear to recover. Alternatively, cell-free passage of the original culture onto fresh detector cells can be used to differentiate these two apparently similar manifestations. The cytotoxicity associated with the structural proteins in the absence of infectious virus would not be expected to pass to the secondary culture.

Giemsa staining may optimize the ability of the operators to visualize certain inclusion bodies (clusters of viral particles) that are characteristic of viral cytopathic effect and is required by 9 CFR 113.53 in assays used to demonstrate that bovine, porcine, equine, and ovine raw materials are free of species-specific viruses.

DETECTION OF HEMAGGLUTINATING VIRUSES

A characteristic of certain viruses (referred to as hemagglutinating viruses) is that one or more of their viral proteins cause hemagglutination of one or more types of erythrocytes. Hemagglutination is an interaction between viral proteins or *hemagglutinins* and erythrocytes, leading to adhesion of the erythrocytes to surfaces, cells, and each other. This property forms the basis of two endpoint procedures that are employed in cell-based viral infectivity assays: *hemadsorption* and *hemagglutination.*

Hemadsorption is performed by adding a suspension of one or more erythrocyte types directly to the monolayer culture of detector cells. If viral proteins of a hemagglutinating virus are expressed from infected cell membranes, the susceptible erythrocytes will bind tightly to the cell membranes. Noninfected cells do not display this binding; therefore, the technique can be used to visualize a focus of infected cells against a background of uninfected cells. For this reason, this particular endpoint may display greater detection sensitivity than other assay endpoints, such as cytopathic effect or hemagglutination. In the advanced stages of infection, binding of erythrocytes to cells, to each other, and to open plastic surfaces in the culture vessel may be observed.

The hemagglutination procedure is performed on the conditioned medium and is essentially an evaluation for free virus or viral hemagglutinins in solution. An aliquot of the conditioned medium from a detector culture is combined, in a microwell plate having v-bottomed wells, with one or more types of erythrocytes. After an appropriate amount of time the plates are evaluated. Absence of hemagglutination is reflected by a well-defined pellet (button) of erythrocytes sedimenting to the bottom of the well. In comparison, hemagglutination is reflected by the absence of a button, or by a button with irregular shape. Scoring the latter represents an opportunity for operator subjectivity. In addition, this endpoint can be considered the least sensitive in detection assays, because it is dependent on achieving a sufficient concentration of viral hemagglutinins in solution. For these reasons, hemadsorption is typically viewed as the more useful and reliable of the techniques for detecting hemagglutinating viruses.

The responses obtained in detection assays using hemadsorption and hemagglutination endpoints are highly dependent on the virus being assayed, as well as on the types of erythrocytes used. Many of the viruses of concern to the biotechnology industry do not cause hemadsorption and hemagglutination, or their hemagglutinins react with red blood cell types not commonly used in detection assays.

DETECTION BY IMMUNOFLUORESCENT ANTIBODY (IFA) STAINING

Certain cell-based viral detection assays are intended to detect specific viral entities and are required for raw materials tests derived from bovine-, porcine-, equine-, and ovine-derived materials (9 CFR 113-53). In order to achieve this, the assays must be optimized with respect to host cell selection, study design, and sample preparation. Specificity of detection is also conferred through use of IFA staining techniques. Primary antisera or monoclonal antibodies directed against the viral antigens of interest are used, either in direct staining applications or in conjunction with a fluorochrome-conjugated secondary antiserum. The immunostained detector cell monolayers are then visualized with an epifluorescence microscope to reveal the presence of reactive infected cells.

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Design of Cell-Based Viral Assays

VIRAL DETECTION ASSAYS

Detector cell cultures are seeded and allowed to incubate for the appropriate amount of time. Viral samples may be inoculated directly into the medium of the mitotic phase of cell culture, but more typically the medium is removed from the overnight detector cell culture and is replaced with the test sample. For the latter method, the test sample must therefore be approximately isotonic, and cytotoxic agents such as selection agents must be maintained within levels tolerable to the detector cell. For test samples comprised of live cells, the sample must be subjected to freeze-thaw before inoculation in order to lyse the sample cells. If the cells are not lysed, a cocultivation involving the detector and sample cells will result. The latter is part of the design for cocultivation assays. But for most of the cell-based infectivity assays such a cocultivation is not intended and could adversely impact the sensitivity of the assay. The test sample is typically allowed to adsorb to the detector cell monolayers for an appropriate amount of time. It is then removed and replaced with the growth medium suitable for the detector cell. Once inoculated, the detection assay involves incubation of the detector cells for a prescribed amount of time, with periodic refeeding as necessary. Endpoint evaluations as described above are performed according to the study design. Variations of the given procedure may be used for exposing detector cells to raw materials. For raw materials, detector cell exposure may consist of incorporation of the test materials into the growth medium used to maintain the detector cells throughout the assay.

VIRAL TITRATION ASSAYS

Viral titration assays are designed to generate quantitative information about the virus of interest. These assays do not quantify absolute numbers of viral particles; rather, the results are expressed in terms of infectious units. An infectious unit is the amount of virus required to establish a productive infection, and several categories of titration assays are used. They vary on the basis of the endpoint used to demonstrate infection and include viral plaque titration or plaque-forming units (units: PFU per mL); 50% tissue culture infectious dose (units: $TCID_{50}$ per mL); and 50% fluorescent antibody infectious dose (units: $FAID_{50}$ per mL). The amount of a hemagglutinating virus in a sample can also be expressed in terms of hemagglutinating titer (units: endpoint dilution, i.e., the greatest dilution of the sample which still results in a positive hemagglutination, or HA, response).

Regardless of the endpoint used, a typical titration assay design consists of a sequence of sample dilutions based on 0.5 $\log_{10}$ or 1 $\log_{10}$ increments. The various dilutions of the sample are then applied to an appropriate number of replicate permissive detector cell monolayers. After a suitable incubation time, the monolayers are scored directly for cytopathic effect ($TCID_{50}$ assay), fixed and processed for immunostaining and scored for reactive cells ($FAID_{50}$ assay), or overlaid with agarose and processed for plaque generation PFU assay. $TCID_{50}$ and $FAID_{50}$ titers are typically calculated using published formulas, such as Spearman-Kärber and Reed-Muench. Assay controls for such quantitative assessments should routinely include a reference sample of known potency.

Detection of Retroviruses

Retroviruses represent a special case for cell-based viral detection assay because of the occurrence of retroviral infection in the absence of responses to the typical endpoints discussed. In order to detect retroviruses, scientists can employ a number of different endpoints. A list of commonly employed indicator cell lines and associated endpoints, and their application in retrovirus detection assays, is provided in *Table 4*.

Table 4. Indicator (Detector) Cell Lines Used in Retrovirus Infectivity Testing

Cell Line	Assay Type	Endpoint(s)[a]	Target Virus
SC-1/XC	Isolation/direct detection	Plaques (XC); RT	Ecotropic murine retrovirus
Balb/C	Isolation/direct detection	Plaques (XC); RT	B-Tropic murine retrovirus
NIH/3T3	Isolation/direct detection	Plaques (XC); RT	N-Tropic murine retrovirus
Mink Lung	Isolation	RT; mink S+L-	Xenotropic, amphotropic murine retrovirus
Mink S+L–	Direct detection	Foci	Xenotropic, amphotropic murine retrovirus
Feline S+L–	Direct detection	Foci	Xenotropic, amphotropic murine retrovirus, gibbon ape leukemia virus (GALV), and RD-114 feline retrovirus
Mus dunni	Isolation, cocultivation	RT; S+L–	Murine retroviruses and retroviruses infectious to humans
QT6[b]	Isolation, cocultivation	RT	Avian retroviruses
RD	Isolation, cocultivation	RT; S+L–	Retroviruses infectious to humans
MRC-5	Isolation, cocultivation	RT; S+L–	Retroviruses infectious to humans
WI-38	Isolation, cocultivation	RT; S+L–	Retroviruses infectious to humans
293	Isolation, cocultivation	RT; S+L–	Retroviruses infectious to humans
A549	Isolation, cocultivation	RT; S+L–	Retroviruses infectious to humans
Raji	Isolation, cocultivation	RT	Retroviruses infectious to humans

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Table 4. Indicator (Detector) Cell Lines Used in Retrovirus Infectivity Testing *(continued)*

Cell Line	Assay Type	Endpoint(s)[a]	Target Virus
Human PBMC	Isolation, cocultivation	RT, HIV p24 EIA	HIV and other human retroviruses

[a] The endpoint assays include the following: RT, reverse transcriptase; PERT, product enhanced reverse transcriptase (including also product enhanced reverse transcriptase and real-time quantitative product enhanced reverse transcriptase assays); and EIA, enzyme immunoassay.
[b] A quail cell; primary fibroblast cultures of chicken or turkey origin are also sometimes used.

Retroviral infection is dependent on the presence of receptors on the host cell membranes. The presence of such receptors confers host cell tropism.

DESIGN FOR RETROVIRUS INFECTIVITY ASSAYS

Two types of infectivity assays are used, depending on the nature of the test material. For materials other than intact cells, the test material is inoculated onto one or more of a variety of detector cells, and the latter are then passaged as required to amplify any virus present. Because of the nature of retroviral replication, cytopathic effects typically do not occur during infection, although there are some exceptions. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus. For test materials in the form of intact cells, detector cells are seeded and subsequently inoculated with the test cells, resulting in a cocultivation. The cultures are passaged five or more times. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus.

ENDPOINT ASSAYS FOR RETROVIRUS DETECTION

Endpoint assays may be classified as direct, which lead to distinct morphological changes in the detector cells; or indirect, as measured by the detection of biochemical, molecular, or immunological markers for infection.

XC-Plaque Assay—The XC-plaque assay was developed as a direct means of detecting infectious murine retroviruses. Detection of the retrovirus is accomplished by UV-irradiating the detector cells used to amplify the virus and overlaying the irradiated detector cells with a specific rat cell (XC). The presence of infectious murine retroviruses in the detector cells is reflected by the formation of distinctive syncytia in the XC monolayer, which are easily visualized when the cultures are fixed and stained with a suitable dye such as crystal violet.

The N/B tropism of an ecotropic murine virus may be determined by inoculating Balb/c and NIH Swiss detector cells with the isolate, performing one or two passages on each cell line, and comparing the XC-plaque titer post passage to that determined for the initial isolate.

Mink and Feline S+L– Focus Assays—The S+L– focus endpoint was developed to facilitate direct detection of infectious murine xenotropic and amphotropic viruses. The test sample may be inoculated directly into cultures of the S+L– cells, or, alternatively, may be amplified first by inoculation into mink lung, human, or *Mus dunni* detector cells. Cell-free supernatants from the detector cell cultures are used to inoculate the S+L– cells. The latter are infected with a sarcoma virus that is replication-defective, requiring the presence of a helper leukemia virus to render it capable of causing transformation of the host cell. The presence of infectious retrovirus virus in the detector cultures is reflected by the formation of characteristic focal areas of cell transformation in the S+L– cells caused by the rescued sarcoma virus.

Detection of Retroviral Reverse Transcriptase—Assays designed to measure reverse transcriptase (RT) activity are useful as an indirect detection method, because the enzyme is indicative of the presence of all retroviruses, whether infectious or not. The RT enzyme is encoded for in the retroviral genome and is used by the virus to transcribe genetic information in viral genomic RNA into proviral DNA.

Radiolabeled Nucleotide Incorporation Assay—The earliest methods for measuring RT activity were based on the measurement of ^{32}P- or ^{3}H-labeled nucleotide incorporation into the complementary cDNA product, using an appropriate RNA template. Incorporation of radiolabeled nucleotide at levels higher than a predetermined threshold is interpreted as evidence of the presence of retroviral RT activity. The contributions of cellular DNA polymerases can be ruled out through use of a dual template assay (having both RNA and DNA templates) or inclusion of activated calf thymus DNA.

Product-Enhanced Reverse Transcriptase (PERT) or Quantitative PERT (Q-PERT)—Polymerase chain reaction amplification has been used to increase the sensitivity of RT activity measurement. RT activity is detected by PCR amplification of complementary DNA, newly synthesized from an RNA template by reverse transcriptase. The assay may be performed with a gel endpoint (PERT) or as a quantitative assay (Q-PERT). This method has increasingly gained acceptance by regulatory agencies. For more details on PCR-based techniques, see the USP general information chapter *Nucleic Acid Based Techniques—Amplification* ⟨1127⟩.

ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) may be used to detect and enumerate viral particles within cells. In addition, the technique allows for differentiation of types A, B, C, and D retroviruses based on morphological considerations and can be used to localize viral particles within the cell. As a technique for identification of viruses (including RNA and DNA viruses in general), TEM of sectioned cells is extremely valuable. The cells, typically sampled during the log phase of growth, are pelleted by low-speed centrifugation, and the cell pellet is fixed with a suitable fixative. The fixed cell pellet is embedded, sectioned, stained, and observed with TEM. Size (diameter) of the particles, morphology, presence or absence of surface features such as envelopes and spikes, and location within the cell can be determined with this technique. Such information is important for the identification of a virus. However, failure to observe viral particles with this method does not conclusively demonstrate the lack of viral contamination in the sample.

Biological fluids may also be evaluated by TEM, primarily to determine particle size and concentration. The cell-free supernatant is subjected to ultracentrifugation to pellet any virus present. The resulting pellet is fixed with a suitable fixative. A

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predetermined number of grid spaces containing representative areas of thin sections of the pellet are evaluated for particles. The enumeration results obtained may show a high degree of variability, and failure to observe particles does not imply that none were present in the sample. Molecular (quantitative PCR and quantitative PERT) endpoints have also been used as alternative methods for estimation of viral particle load in samples.

ANTIGEN-CAPTURE ENZYME IMMUNOASSAY

Specific viral proteins (e.g., HIV p24 antigen or avian leucosis viral envelope proteins) may be detected as a means of determining the presence of a retrovirus. Viral antigens are captured by specific antibodies coated onto microtiter plate wells and are detected by the addition of a second labeled antibody and appropriate substrate.

Assays Designed to Detect Specific Viruses

Additional methodologies have been developed to allow detection of specific viruses or groups of viruses. These types of assays are often used for raw material evaluation. In some cases, these specific assays were developed because the target viruses do not cause endpoint responses in the viral screening assays. In contrast to screening assays, specific virus assays are typically optimized for detection of the target virus or viruses. This optimization takes into account the lability of the virus, the host range, the possible endpoint responses elicited, and any special requirements of the target virus. The use of well-characterized viruses as positive controls in such assays provides assurance that the methodologies are suitable for the target virus or viruses. Spiking of the test sample matrix with the positive control virus enables the investigator to assess the potential for matrix interference and to assess the limit of detection for the method. Such considerations are not applicable to screening assays. Specific virus testing for bovine- and porcine-derived raw materials is discussed below. Evaluation of caprine, ovine, equine, canine, and feline raw materials is also stipulated in 9 CFR section 113.47. This section should be consulted with respect to the viruses of concern, and 9 CFR 113.52 should be consulted for methodology. A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in *Table 2*. A list of commonly employed indicator cell lines and their application in detection of specific viruses is also provided (see *Table 3*).

DETECTION OF BOVINE VIRUS CONTAMINATION

Raw materials of bovine origin include such commonly employed medium components as fetal bovine and calf serum, serum albumin, collagen, thrombin, and trypsin. Each of these additives represents a route of entry for adventitious viral contaminants into a cell culture or manufacturing process. Requirements for evaluation of such materials ensure the absence of contaminating viruses.

For the details on testing for bovine serum and its derivatives, see future general chapter *Bovine Serum* ⟨1024⟩.

DETECTION OF PORCINE VIRAL CONTAMINANTS

Raw materials of porcine origin include trypsin as well as other cell culture reagents. The specific porcine viruses of concern in the United States are stipulated in 9 CFR 113.47 and include porcine parvovirus, porcine adenovirus, transmissible gastroenteritis virus, and porcine hemagglutinating encephalitis virus. In addition, porcine raw materials must also be evaluated for the presence of bovine viral diarrhea virus (BVDV), reovirus, and rabies virus. Porcine tissues intended for xenotransplantation into humans also are routinely evaluated for the porcine endogenous retrovirus (PERV). The host cells typically used in the detection of porcine viruses are porcine testicle or porcine kidney, a bovine cell, and Vero cells. The methodology described in 9 CFR 113.52 is analogous to that for evaluation of bovine raw materials and includes provision for multiple subcultures, for Giemsa staining of fixed cells, for hemadsorption testing, and for use of specific immunostaining of fixed cells.

CELL-BASED DETECTION OF MURINE MINUTE VIRUS

Murine minute virus (MMV) is a mouse parvovirus that has been detected in biologics manufacturing involving Chinese hamster cell substrates. As with other parvoviruses, MMV represents a special case in that the virus is difficult to inactivate using typical cleaning agents and is capable of surviving for prolonged periods of time on surfaces. Cell-based assays for MMV involve detector cell lines that are especially susceptible to this virus, such as 324K (a human cell) and A9 (a murine cell). Optimization for detection of a parvovirus also includes provision for detector cell subcultures to remain in log-phase division for a significant portion of the incubation period. Endpoints for detection of MMV include one or more of the following: cytopathic effect, hemagglutination of mouse and guinea pig erythrocytes, immunostaining, and polymerase chain reaction.

CELL-BASED DETECTION OF INSECT-BORNE VIRUSES

Insect-borne viruses include both viruses infectious only for insect cells (e.g., baculovirus) and those transmitted to mammalian cells via insect vectors (arboviruses). Detection of the former may be accomplished using an insect cell as a detector cell. Suitable substrates might include cells of *Spodoptera, Trichoplusia, Drosophila,* mosquito, or other insect origin. Such cells are typically cultured at lower temperatures (25° to 28°) relative to mammalian cells, and many of these cultures are suspension or semiadherent at best. Endpoints may include cytopathic effect, electron microscopy, and PCR.

Of more relevance to patient safety is the detection of arboviruses (insect-borne viruses infectious to animals and humans). This may be accomplished using a suitable mammalian detector cell. The Syrian hamster kidney cell (BHK-21) is a cell line that has shown susceptibility to a wide range of arboviruses. This cell line grows in a monolayer culture, and the endpoints that may be used include cytopathic effect, hemadsorption and hemagglutination, and PCR.

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CELL-BASED DETECTION OF HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (CMV) is a slow-growing virus of special concern for biologics produced using human cell substrates. It may be detected in cell-based assays using human diploid detector cells such as WI-38 or MRC-5, provided that sufficiently long durations of incubation are employed (28 or more days). The endpoints include cytopathic effects and immunostaining and/or PCR.

In Vivo Methods

Intact and susceptible animals may serve as potential host organisms for detecting viruses in test samples. In this case, viral proliferation in the tissues of the host animal may be reflected as adverse health effects (including death) that can be monitored and recorded. Viral detection assays based on intact animals are intended to complement in vitro assays, because some viruses that do not cause a response in the in vitro assays may be detectable in the animal systems (and vice versa). Viral safety studies employing live animals must be performed in accordance with applicable regional guidelines for the ethical use of animals, using laboratories that are accredited for the housing of the animals.

IN VIVO VIRAL SCREEN

The in vivo viral screen is used primarily for cell bank, viral seed stock, and viral vaccine testing and is considered to complement the in vitro virus screening assay. Multiple animal species, as well as multiple injection routes, are employed to provide a broad range of host tissues and possible responses. A list of commonly used host animals, routes of inoculation, and target viruses are shown in *Table 5*.

Table 5. In Vivo Viral Screening Assays

Host Animal	Route of Inoculation	Target Virus
Suckling mouse	Intraperitoneal injection	Arboviruses
	Intracranial injection	Coxsackie A and B
	Per os injection	Herpes simplex Type 1 and 2
		Togaviruses
		Junin
		Herpes B
Adult mouse	Intraperitoneal injection	Rhabdoviruses
	Intracranial injection	Togaviruses
	Per os injection	Lymphocytic choriomeningitis virus (LCMV)
Guinea pig	Intraperitoneal injection	Rhabdoviruses
	Intracranial injection	LCMV
		Lassa
		Junin
		Marburg
		Ebola
		Vaccinia viruses
Embryonated hens' eggs	Allantoic	Arboviruses
	Yolk sac	Equine encephalomyelitis viruses
	Chorio-allantoic membrane	Herpes viruses
		Influenza
		Mumps
		Newcastle disease
		Parainfluenza Types 1 and 2
		Rabies
		Vaccinia
		Variola
		Lymphogranuloma venereum
		Ornithosis

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Following injection of the test sample, each animal model is monitored for an appropriate period of time that allows for the observation of clinical signs of viral infection. Any abnormality is investigated to determine the cause of the effect.

The suckling mice are observed for an appropriate period of time. Pooled homogenates from any surviving animals are then passaged into additional litters of suckling mice. The latter are observed for an additional period of time.

The guinea pigs are observed for clinical signs of viral infection and for injection site lesions. Necropsy for gross tubercular lesions is performed for certain types of test samples.

Allantoic fluids from eggs can be tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Additional fluids are pooled for each treatment group (test article and control), and these are passaged (inoculated) into a new group of embryonated eggs. Following an appropriate incubation period (typically measured in days), the allantoic fluids are again tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Following injection by the yolk sac route, the eggs are incubated for at least 9 days and are assessed for viability. The yolk sacs are then harvested and pooled for each group (test article and control), and a 10% solution of the resulting material is inoculated by the same route into a new group of embryonated eggs. The eggs are again incubated for an appropriate period of time (days) and are assessed for viability.

IN VIVO ASSAYS INTENDED TO DETECT SPECIFIC VIRUSES

Some in vivo assays are designed to detect, if not specific viruses, at least specific sets of viruses. The antibody production assays use the production of a humoral immune response in susceptible host animals inoculated with test samples. Viral antibody-free animals of the various species are injected with the test sample. At the end of an appropriate incubation period, one or more of a variety of endpoint assays may be performed to detect the generation of a humoral antibody response in the animal sera. Production in the animal of antibodies directed against a specific virus provides evidence of the presence of viral antigen or infectious virus in the test sample. This type of assay is typically used to ensure that rodent cell banks and viral seed stocks are free of adventitious viruses. Three antibody production assays, along with the route of injection and target viruses, are summarized in *Table 6*.

Table 6. In Vivo Antibody Production Assays

Antibody Production Assay	Route of Injection	Target Virus
Mouse antibody production (MAP) assay	Intranasal	Ectromelia
	Intraperitoneal	Hantaan
	Intracranial	Mouse K
		Lactate dehydrogenase elevating virus
		Lymphocytic choriomeningitis virus (LCMV)*
		Murine minute virus
		Mouse adenovirus
		Mouse cytomegalovirus
		Mouse encephalomyelitis virus type II
Mouse antibody production (MAP) assay		Mouse hepatitis virus
		Epizootic diarrhea of infant mice
		Pneumonia virus of mice
		Polyomavirus
		Reovirus type 3
		Sendai
		Mouse thymic virus
Hamster antibody production (HAP) assay	Intranasal	Lymphocytic choriomeningitis virus (LCMV)*
	Intraperitoneal	Polyomavirus
	Intracranial	Reovirus Type 3
		Sendai
		Simian virus 5

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Table 6. In Vivo Antibody Production Assays *(continued)*

Antibody Production Assay	Route of Injection	Target Virus
Rat antibody production (RAP) assay	Intranasal	Hantaan
	Intraperitoneal	Kilham rat virus
	Intracranial	Mouse encephalomyelitis virus type II
		Polyomavirus
		Reovirus type 3
		Sendai
		Toolan's H1 virus
		Rat coronavirus/sialodacryoadenitis virus

* A group of test sample–injected mice is challenged with a known lethal dose of authentic LCMV. If this group of mice does not die from the challenge dose, a second group with twice the number of mice is used for a repeat challenge. If there are survivors in this group, the test sample is considered positive for LCMV.

Considerations for Validation, Matrix Qualification, and Quality Control of Cell- and Animal-Based Test Systems

Viral detection assays used to ensure the viral safety of human and animal therapeutics are expected to have undergone validation. The approach to the validation depends on the nature of the assay and associated regulatory compliance level.

Any assay should be sufficiently developed that it can be performed with an appropriate set of predetermined system suitability and acceptance criteria. These criteria usually include the use of relevant negative and positive controls but may also include requirements for linearity and meeting of a predetermined detection limit. The results constituting a positive or negative response in the assay should be established prior to execution of the validation. An assay used under GMP compliance is expected to have been validated according to appropriate guidelines.

An assay used to ensure the safety of a commercially marketed biological product must be further characterized for suitability in the presence of the specific product matrix. The matrix qualification study should address the potential for specific interference with the viral detection endpoints used in the assay, and typically involves spiking of one or more model viruses into the product matrix at levels approaching the limit of detection to ensure the absence of interference.

For quantitative detection assays, the detection limit should be probed. This usually involves spiking of the model virus(es) at decreasing amounts into medium or the product matrix. The lowest spiking level of the virus reliably detected is used as an approximation of the actual limit of detection of the assay. Experimental error for these cell culture-based assays is usually expected to be in the range of 0.5 to 1 $\log_{10}$. The determination of a detection limit is less meaningful for limit tests and viral screening assays in general. For the latter, knowledge of the detection limit for one virus does not imply a similar limit for another virus. Since screening assays are not optimized for a specific virus, the limit of detection for the assay can vary greatly from one virus to another.

Animal-based viral detection systems are generally not subject to the requirements for validation, matrix qualification, use of positive controls, and determination of detection limit that regulatory agencies expect of cell-based and biochemical tests. The use of animals for safety testing is subject to the regional guidelines for the ethical use of animals, and the kinds of activities listed above generally are not considered appropriate use of animals. However, negative control animals are included in these assays, and retrospective validation or gap analysis based on historic incidence of system suitability failures or positive findings is sometimes possible.

DETECTION OF VIRAL COMPONENTS

Direct detection of viral components can provide a direct measurement of viral levels in a sample preparation. It has also become primarily important for detection or identification of viruses in biological products or in the raw materials used in their manufacture. Systems capable of identifying components unique to specific phases associated with viral latency and replication are now available. During interaction with their host cells, viruses may incorporate modified host molecules during the production of new intact virus particles, or they may induce discernable changes in host cell makeup or function.

Most immunological methods and reagents currently available detect the constituents of intact virions. It is the relative abundance of these proteins that makes them most amenable to the development of antibody-based reagents. Abundance also makes them optimal targets for detection of the virus. Recently developed targeting and detection reagents are aimed at minor viral components that may be found only during specific phases of replication. These allow a more detailed analysis of the stage of viral infection. The basic methodology for the detection of viral antigens is well established, but more recent innovations in materials and reagent development have broadened its application.

Developments in the targeting and detection of viral nucleic acid components have led to enzyme-based systems for the amplification of nucleic acids in vitro and in situ (see *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩). The potential specificity of this detection method allows the examination of biological systems with a high degree of confidence for the presence or absence of a specific targeted virus. A wide variety of reagents, technology platforms, and methodologies are available. The aim of this section is to elaborate on the most common practices and platforms used in the detection of viral components.

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Sample Selection and Preparation for the Detection of Viral Components

This subsection addresses general considerations for various types of test samples and the most common assay targets (viral proteins and nucleic acids). The target proteins may have varying levels of posttranslational modification (e.g., glycosylation, phosphorylation), and the target nucleic acids may be either RNA or DNA, single or double stranded. Therefore, it is important to have a basic understanding of the physicochemical nature of the virus under study so that the sample handling procedures support the detection of the target component. For detailed considerations regarding the extraction of nucleic acids, see the USP general information chapter *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩.

Types of Samples

CELLULAR

When viral components are associated with intact cells, samples must be generated either as whole cell lysates or as subcellular fractions. Maintaining the temperature of the test samples at or near freezing (0° to 4°) during processing and limiting the time that samples are held in an unfrozen state will reduce the potential for loss of target antigens and nucleic acids due to cytosolic enzymes (proteases, nucleases) present in the cell lysates. Reagents that can inactivate or limit the activity of such enzymes may be used to prevent degradation of the target components, especially when exceptionally labile samples must be handled at room temperature. Centrifugation of intact cells allows for some additional manipulation of the sample matrix. The growth medium can be discarded and the cells suspended in a buffer formulated to enhance the recovery and detection of the targeted viral component. Collection and storage parameters should also account for the presence of cellular DNA. This can increase the viscosity of the sample, rendering it difficult to pipet.

TISSUE CULTURE SUPERNATANT

Depending on the stage of the infection and the type of virus involved, the conditioned medium may represent a preferred test sample. An advantage is that the presence of cellular debris can usually be reduced through use of a low-speed centrifugation (clarification) step. The main disadvantage is the potentially low concentration of the analyte, and therefore concentration of the sample may be required.

PROCESS INTERMEDIATE (UNPROCESSED BULK HARVEST)

In general, viral safety lot release testing is done at the bulk harvest stage prior to any purification. This is true regardless of whether the assay detects infectious virus or viral components. The presence of host cell DNA may need to be assessed in the case of biologics manufactured in animal cells.

Sample Stability and Matrix Effect

Sample stability is a key element in the successful detection of viral components. Protein structure can be altered by numerous environmental factors, including pH, ionic strength, solvents, detergents, temperature, and free radicals. In addition, complex biological matrices frequently contain proteolytic enzymes that can alter or destroy key antigenic features of a protein or peptide. Sample collection, storage, and handling must allow maintenance of the antigenic features targeted by reagent antibodies.

Conformational changes affecting the opportunity for antigen detection are difficult to address. Depending on the reagents required for detection, conformational changes may be required for antigen detection. For example, if antibodies are produced for an antigen detection system using native viral antigen, then unmasking and maintaining the conformation of the antigen throughout the sample preparation is essential. Conversely, if peptide fragments are used to produce antibody, then a denaturation step may be required to allow for effective antigen detection.

Conditions associated with sample preparation must be investigated in a combinatorial fashion whereby one parameter or component is varied while all others remain fixed. In this way, the formulation of lysis and processing buffers can be optimized for pH, ionic strength, and types of detergents and denaturants. The sample preparation steps must condition the targeted antigen in order to obtain the form most readily recognized by the reagent antibody.

The stability of nucleic acids in test samples is largely affected by nuclease activities present in the sample and the degree of protection provided by the intact structure of the virus particle. Encapsidated nucleic acids are particularly stable as long as the integrity of the capsid is maintained. Viral capsids are vulnerable to proteolytic digestion. Virus particles stored at ambient temperature as part of a complex biological matrix are especially susceptible to degradation by proteases. Storage at refrigerated temperatures (2° to 8°) for short periods of time or at temperatures below freezing can be used to limit proteolytic activity. When samples are stored at frozen temperatures, freeze-thaw cycles should be limited. Under conditions where an individual sample must be accessed multiple times, preparation of aliquots is advisable.

SAMPLE COLLECTION

In a biotechnology setting, sample collection is dictated by sampling plans that are established to meet regulatory requirements. Nucleic acid testing in association with an amplification step has the potential of detecting a virus at the earlier stages of infection. The use of nucleic acid amplification methods reduces the dependence on timing and the amount of material required, because the amplification process effectively boosts assay sensitivity by increasing the amount of target relative to background.

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General aspects of nucleic acid sample preparation and stability are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. The following section specifically addresses the unique aspects of viral sample selection and preparation.

SAMPLE STORAGE

Conditions for sample storage should be consistent with maintaining the antigenic properties of targeted viral proteins and/or preserving the nucleic acid content of the sample. The duration and temperature of storage is dictated also by cycle times associated with testing.

IMMUNE COMPLEX DISRUPTION

The masking of antigen epitopes may occur when other proteins associate at or near the epitope targeted by reagent antibodies. For viral antigens this may occur when the antigen comprises the structural component of the virus. Such viral antigens are likely to retain strong affinities for other viral proteins or the ability to exist in multimeric form under normal conditions for detection. Another obstacle for epitope recognition is the naturally occurring immune complex when reagent antibodies have been developed to detect native protein in a blood plasma matrix. Immune complexes consisting of viral antigens and host antibodies are normal in such physiologic samples. The stronger the host immune response, the more likely masking of antigen due to immune complex formation will occur. Methods aimed at the preparation of blood and plasma samples for detection should address the presence of preexisting immune complexes and incorporate steps designed to disrupt such complexes to improve the opportunity for viral antigen detection.

Detection of Viral Antigens

Viral capsid proteins are common targets for antigenic detection methods. Structural proteins that make up the framework of the viral core are often some of the most abundant viral proteins produced during viral replication. In nonenveloped viruses, the core structural proteins are likely to provide the dominant antigenic features. When the virus is enveloped, proteins associated with the envelope often provide key antigenic features. This section examines methods commonly used to detect viral antigens and addresses considerations aimed at optimizing formation of the appropriate immune complex.

ASSAYS USED FOR THE DETECTION OF VIRAL ANTIGENS

Immunologic methodologies used to detect viral antigens are based on the specificity and affinity of the antibody and viral antigen interaction. Of the various platforms available, one commonly employed in viral safety testing is immunofluorescent antibody staining. This lends some degree of viral specificity to the cell-based methods described in the first part of this chapter. Other techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and Western blotting. The principles and general methods for these assays will be described in the USP general information chapters *Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Reagent Development* ⟨1103⟩, *Immunological Test Methods—Immunoassay Methodologies* ⟨1104⟩, and *Immunological Test Methods—Assay Design, Quality Control, and Data Analysis* ⟨1105⟩, being prepared for future publication. The following sections will generally address aspects specific to their use in virology.

Immunofluorescence Assay—The immunofluorescence assay (also referred to as immunofluorescent antibody staining) is used to detect viral proteins expressed in various cellular compartments. Since the technique can detect viral antigens within single cells, it confers a high degree of sensitivity and enables infection to be detected at a very early stage. The technique is often employed as a detection endpoint for cell-based viral infectivity assays to provide additional sensitivity and specificity (see tests for raw materials as described in 9 CFR 113.53). In addition, the technique is employed for verifying the identity of viral stocks and is useful as a means of identifying viruses detected in viral screening assays.

Enzyme-Linked Immunosorbent Assay (ELISA)—ELISA is best suited for detection of soluble antibodies and antigens in a variety of test samples. Sensitivity, quantification, robustness, ease of experimentation, and readily available inexpensive reagents make it adaptable to a high throughput environment. For viral antigen detection, a sandwich ELISA assay is commonly used. A viral antigen specific antibody (preferably a monoclonal antibody with high affinity) is first immobilized onto a solid phase. The test sample is then incubated for a predetermined period under appropriate conditions. After washing, a second antibody that recognizes the viral antigen is incubated. The second antibody is linked to either an enzyme or a chromomeric reagent that emits signal with an appropriate substrate and can be recorded with an appropriate instrument. The assay can be performed in a qualitative or a quantitative manner. For a qualitative ELISA assay, sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample that is equal to or greater than the cut-off value is considered positive. For a quantitative ELISA assay, an additional standard curve with a positive reference standard material of known quantity must be established. The number of replicates should be adequate to determine the assay variation and linearity. The quantity of test sample can be calculated against the standard curve.

Radioimmunoassay (RIA)—The radioimmunoassay is a versatile quantitative immunoassay that can be used to detect substances including viral antigens and antibodies. It even can be applied to nonprotein molecules as long as an antibody that specifically binds the test substance is available. Radioimmunoassays can be customized in different formats to suit specific test requirements. Many of the considerations taken into account with other immunological assays are applicable to the RIA. In general, radioimmunoassays can be divided into two major categories: solution (homologous) and solid-phase radioimmunoassays. Both methods have been successfully used to detect and quantify a variety of viral antigens or components of viruses, such as hepatitis A, B, and C; human and murine retroviruses; adenovirus; avian C-type virus; rubella virus; and respiratory syncytial virus.

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Western Blotting (Immunobloting)—Western blotting, also known as immunoblotting, is used to identify specific antigens in the presence of other, potentially cross-reactive antigens. In this case, the specificity required is obtained by combining the antigen-antibody reaction with some form of separation (typically electrophoresis). Depending on the visualization methods employed (including digital methods such as densitometry), this method can be quite sensitive and even semiquantitative. One advantage of this approach is that in addition to detecting the antigen using an immunological approach, data on the approximate mass of the target protein may be obtained. A potential caveat is that most test proteins are denatured during this procedure and that antibody that depends on epitope conformation may not recognize the linear epitopes.

Detection of Viral Nucleic Acids

The detection of viral nucleic acids provides another route for the determination of viral loads and for establishing the identity of a contaminant. Nucleic acids, like protein antigens, are essential components of viruses, and detectable quantities are usually indicative of viral presence. Detection assays can be designed and developed in some cases to parse viremia into phases, especially when the differentiation of nucleic acids along functional forms and configurations can provide clear insight into viral activity. Assays can be designed to determine whether viral DNA has been integrated into the host genome or still is encapsidated. Early viremia may be detected as viral mRNA transcripts prior to the accumulation of detectable viral particles. Nucleic acids may be the only detectable viral component of viruses that do not replicate well in tissue culture systems. Such systems may fail to produce mature virus particles, but the detection of viral transcripts can provide insight into whether the virus has the ability to infect the cell. Nucleic acid testing represents the most useful endpoint for the detection of certain viruses failing to cause responses using typical endpoints.

SAMPLE PREPARATION: SPECIAL CONSIDERATIONS FOR NUCLEIC ACID TESTING

The degradation of nucleic acids in samples can be limited through proper handling and storage practices and even enhanced by closely linking sample collection and preparation steps. In addition to preparing nucleic acids for further processing, denaturation is an important step toward stabilizing nucleic acids where storage temperatures extend above 0°.

Denaturation and Dissociation of Virions (Viral Lysis)—Chaotropic detergents and salts can be important agents for disrupting and removing viral proteins that make up the viral capsid. Their addition can provide a useful first step when concentration of virions is not necessary or even possible. In sufficient quantity they rapidly denature the entire contents of a biological sample, essentially fixing nucleic acid content through the inactivation of nucleases and other proteins that may affect sample stability. Saturated solutions containing guanidium salts, such as guanidine hydrochloride or guanidine isothiocyanate, are commonly used for the dissociation of viral nucleic acids from protein components. These solutions may be used alone or in combination with ionic detergents and other denaturants such as phenol. The main advantage of guanidinium salts is that they are readily removed during the concentration of viral nucleic acids using ethanol or isopropanol. Urea may also be used as a mild denaturing agent, although it does not perform as effectively as guanidine in its ability to disrupt virus particles.

Deproteinization—The removal of proteins during the processing of samples for the detection of viral nucleic acids is helpful in ensuring the reproducibility and robustness of assays, particularly those that rely on amplification to detect exceptionally low quantities of nucleic acids. Several strategies may be used to facilitate deproteinization of the sample; they are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩.

Recovery of Viral Nucleic Acids—Separation and recovery of extracted viral nucleic acid are important steps in the testing of nucleic acids. Nucleic acid yield and purity obtained at this step are critical determinants of assay robustness. Poor nucleic acid recovery and limited purification may inhibit amplification and detection reaction resulting in poor assay sensitivity. The development of high-yield, high-purity recovery steps is an important goal in the optimization of nucleic acid detection methods. Details on the general aspects of extraction and detection of nucleic acids are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. However, if no inhibitory components are identified in the system, direct testing on an aliquot of a sample may increase the assay sensitivity.

Isolation of Viral DNA—Integrated viral genomes must be recovered and processed along with the genome of the host cell. The viral genome is analyzed within the context of the host genome, and therefore similarities between virus and host genomes must be accounted for to ensure that assay results are specific for the virus and do not simply reflect the presence of cross-reacting host genome sequences. Viral genomes that exist as episomal entities require similar consideration, but there may be opportunities during sample preparation to limit the amount of host nucleic acid present in the preparation. Sedimentation gradients or silica-based separation chromatography can sometimes be used to enrich episomal nucleic acids through size-related exclusion or partitioning of processed nucleic acids.

The recovery of encapsidated viral genomes may allow for larger amounts of material, especially if the target virus has accumulated in large quantities in the system being monitored. When the amount of material available for recovery is low or the processing of large amounts of the material is impractical, the process of isolating and conditioning the virus particles, and subsequently the encapsidated genome, must be compatible with the assay system that will be used. Portions of the viral genome may need to be amplified, or the genome itself may be captured in an elaborate process that allows detection through the generation of an amplified signal. A standard method for isolating viral genomes may need to be modified appropriately to ensure that materials used in the preparation do not interfere with steps conducted later in the process.

Viral genomes that consist of RNA are typically converted to a DNA intermediate that is easier to handle and store. Methods aimed at the isolation of viral genomes consisting of RNA, however, require similar considerations concerning the localization of the genome: replicating RNA viral genomes may be recovered as part of a preparation of host total RNA or even mRNA if the genome contains polyA sequences. RNA lends itself more effectively to hybrid capture methods during isolation, and hybrid capture can be used to enrich RNA preparations specifically for RNA viral genomes. RNA genomes can be extracted from virus particles in much the way that DNA viral genomes are extracted. RNA genomes are converted to complementary (cDNA) sequences using retroviral RT. Storage is of greater concern for naked viral RNAs. Storage of RNA usually requires temperatures below –20°.

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Detection of Viral Genome Versus Viral Transcripts

Viral genomes exist as either DNA or RNA, or sometimes both: in the case of retroviruses the integrated genome is DNA, whereas the encapsidated form is RNA. The ability to differentiate among the various forms of viral nucleic acids can help to elucidate the course of specific viral infections. Assays for nucleic acid activity can differentiate readily between integrated and encapsidated genomes when the form of the viral nucleic acid varies between states, as in the case of retroviruses. Incorporation of specific nucleases into the assay methodology can be used to reduce or eliminate one form over the other. If viral genomes are known to integrate at specific sites within the host genome, primers and probes can be developed around the integration site and incorporate significant elements of both host and viral genomes. Some viral mRNAs contain splice sites, and the differentiation of spliced nucleic acid sequences from unspliced sequences creates a unique mechanism for determining the status of nucleic acid localization and infection.

CHARACTERIZATION OF DNA VIRAL GENOMES

Methods for the recovery and preparation of viral genomes for characterization depend on the state of the viral genome. If the genome has been incorporated into a cellular compartment, the recovery and preparation strategy must take into account the cellular components that make up the sample matrix. If the viral genome targeted for analysis is the encapsidated form, the methods must focus on recovery of the virus particle and must include additional steps aimed at extracting the nucleic acid from the individual particles. Identification and characterization of viral genomes require specific complementary nucleic acid probes and primers whose sequence will be dictated by available information about the sequence of the targeted viral nucleic acids and the type of assay that will be used. Determination of the sequence of the viral nucleic acid of interest usually provides the most unambiguous means for characterization. However, a number of methods can be used as simple indicators for the presence or absence of specific sequence-based characteristics. For example, melting curve profiles using short oligonucleotide sequences can be used to establish whether a specific viral genotype is present.

IDENTIFICATION AND GENOTYPE ANALYSIS

Nucleic acid testing is often used to identify viral isolates obtained from viral screening assays or to provide identity for viral stocks. Methods used for the identification of viral genomes are not unique to other applications in the field of molecular biology. Typically, an amplification step is required in order to achieve quantities for analysis. Amplified sequencing of the amplicons or application of a standard hybridization technique may be employed for more detail as to the nature of the amplified signal. For more details, refer to *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

HYBRIDIZATION TECHNIQUES

A variety of hybridization techniques are used to detect viral nucleic acid sequences, including Southern blot, Northern blot, DNAse/RNase protection, in situ hybridization, microarray technology, and other techniques. The description of these methods, which is well beyond the scope of this chapter, can be found in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩.

DETECTION OF ANTIBODIES TO VIRAL ANTIGENS

A variety of methods are available for detection and quantification of antibodies to viral agents, including neutralization, complement fixation, and immunoassays based on enzyme- or fluorescently labeled reagents. Although many details of the immunological methods mentioned above are beyond the scope of the chapter, this section addresses specific application aspects of viral antibody detection, including preparation and storage of test samples, common assay methods and platforms available, and specific examples of how these assays may be used to measure antibodies to specific viral agents.

Viral Structure Relative to Antigenic Composition and Selection of Antibody Assay

Mammalian viruses vary considerably in their nucleic acid content and thus the number of antigenic, virus-specific proteins produced.

Many viral proteins or glycoproteins are highly antigenic and induce a potent humoral immune response during natural infection, whether in humans or in animal models. In most cases, the immune system responds when the virus and its antigens appear in the extracellular fluid or on the infected cell membranes. The degree to which viral antigens are expressed is governed by the intracellular replication and protein synthesis of viruses in host organ tissues and by the several possible types of virus–host cell interaction. Antibodies produced as a result of natural viral infection are likely to represent the broadest response to antigens in their native state.

When selecting, developing, or evaluating an assay method for measurement of antibodies to viral proteins, the analyst must take into account the source of the antibodies and the method by which they were obtained or prepared. Details will be presented in *Immunological Test Methods—Reagent Development* ⟨1103⟩ and *Immunological Test Methods—Immunoassay Methodologies* ⟨1104⟩.

General Considerations Regarding Sample Preparation for Antibody Detection of Viral Antigens

Antibodies are relatively stable, but care must be taken to ensure the integrity of the test antibodies during sample preparation and storage. Serologic tests may be developed to measure antibodies to viral agents in unfractionated biological fluids. The possibility for matrix interference with the antibody detection method should be considered.

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In general, biological test samples should be clarified by centrifugation or filtration, depending on their intended use. Serum samples should not be hemolyzed, lipemic, or icteric. In some cases the specimen should also be heat-treated to inactivate endogenous complement and other components.

Test samples should be processed as soon as possible. When it is necessary to store samples, most test samples should be stored at –20° for short-term storage and below –80° for long-term storage. For all samples, the stability of the material needs to be assessed experimentally. Aliquots of appropriate volume should be prepared in accordance with test procedures to avoid unnecessary freeze-thaw cycles.

Antibody Methods

This section discusses the primary methods for detecting antibodies directed against viral antigens. The assay methods often include a variety of alternative formats for the detection of antibody. Only the more commonly used formats for antibody detection are discussed in this section. Some methods, including fluorescent antibody assays and enzyme immunoassays, are widely applicable to the detection of antibodies to many different viral agents; others are limited to selected viruses having certain properties (e.g., hemagglutinins).

IMMUNOFLUORESCENCE MICROSCOPY FOR ANTIBODY DETECTION

When fluorescein isothiocyanate (FITC) is chemically coupled to an antibody molecule, the resulting *FITC-labeled antibody* can be used as a secondary antibody probe to detect the presence of a primary, virus-specific antibody bound to a virus-infected cell on a microscope slide (indirect immunofluorescence).

The indirect immunofluorescence or indirect fluorescent antibody (IFA) assay is one of the most basic and useful methods for detection of antibodies to viruses. The assay can be used to detect both virus-specific IgG- and IgM-class antibodies. When the assay is used to detect IgM antibodies, it usually requires the physical removal or inactivation/binding of IgG-class antibodies. In the absence of this step, the presence of IgM-specific antibody may be masked by excess IgG-specific antibody competing for primary binding sites on the substrate surface. IFA assays may be qualitative or quantitative.

The IFA for antibody to viral agents requires the use of virus-infected cells expressing viral antigens in cellular membranes. Viral stocks are prepared, titered, and used to infect permissive cells in tissue culture. The cells are harvested at appropriate times, washed, and spotted onto multiwell microscope slides at an appropriate density. Control slides are also prepared with noninfected cells. The slides are allowed to air-dry and then fixed in cold acetone. The fixed slides can then be stored under appropriate conditions for extended time periods. The stability of the viral antigens over time should be confirmed.

The test article to be examined for the presence of virus-specific antibodies can be applied to the slide, followed by an appropriate secondary antibody conjugated with a fluorescent tag that can be visualized under a fluorescent microscope. IgG- or IgM-specific antibodies can be distinguished by using the appropriately prepared secondary antibody.

Reading and correctly interpreting endpoints of IFA slides for antibody detection requires an experienced analyst, particularly when cellular location and fluorescent-staining patterns are critical for a specific virus. Such interpretation requires the use of appropriate controls and scoring or intensity of fluorescence. This is highly dependent on the quality of reagents, the consistency of the fluorescent microscopy and light source being used, and the experience of the analyst.

ENZYME IMMUNOASSAY FOR ANTIBODY DETECTION

The EIA and variations of it are the most widely used methods for the detection of viral antibodies in serum and other biological products. The most commonly used EIA for antibody detection is referred to as a noncompetitive solid phase EIA for antibody detection. The typical configuration of an EIA for antibody involves coating tubes or microwell plates with viral antigen(s), the addition of test serum or product to the tubes or wells, the binding of specific antibody in serum or product to antigen, and detection of bound antibody by addition of a second antibody with binding affinity to the primary antibody, which is labeled for its detection.

Assays can be specific to IgG- or IgM- class antibody or may detect total antibody. Assays for IgM may achieve improved when performed as *IgM-capture* assays. These assays involve the use of plates or wells coated with anti-IgM antibody to total IgM in serum or product as a first step. Subsequently, viral antigen is added; it binds to the plate only if specific IgM antibody has initially been captured, and it is detected by addition of a second labeled antibody specific to the antigen. The assay is most often performed as a qualitative measure of the presence of an antibody for a specific virus. Sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample equal to or greater than the cut-off value is considered positive.

COMPLEMENT FIXATION TEST

Complement fixation has selective value in allowing for simultaneous assay of antibodies to a wide variety of viral agents. The procedure involves multiple variables consisting of two pairs of antigen–antibody reactions. The first reaction, between a known virus antigen and a specific antibody in the test sample, takes place in the presence of a predetermined amount of exogenous complement. The complement is removed by the antigen–antibody complex. The second antigen–antibody reaction consists of sheep red blood cells (SRBCs) and hemolysin (antibody against SRBC). When this indicator system is added to the reaction mixture, the sensitized SRBCs will lyse only in the presence of free complement. The extent of lysis of SRBCs is inversely correlated with the amount of the antibody in the test article.

The experimental procedure involves the optimal titration of concentrations of hemolytic serum, complement, and viral antigen, using chessboard format. If used as the test sample, human serum should be inactivated at 56° for 30 minutes to inactivate the endogenous complement activity. A number of important controls must be run along with the test, and results must be within limits before the test can be properly interpreted. These include the sensitivity of SRBCs to lysis and complement

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concentration used. The relative amount of virus-specific antibody present can be determined by testing serial dilutions of the serum or product. The *complement-fixing titer* is the reciprocal of the highest dilution that prevents 50% hemolysis.

NEUTRALIZATION FOR ANTIBODY DETECTION

Neutralization for the measurement of antibodies to viral agents is still one of the most valuable assays available because of its high specificity and its ability to detect neutralizing antibodies. Neutralization is defined as the loss of viral infectivity through the binding of specific antibodies to viral coat proteins (or envelope glycoproteins) on the surface of the infectious viral particle. The assay may be used to measure the presence of antibodies to a known virus in a serum or product sample, or conversely to identify an unknown virus by using a serum or product sample containing known antibodies.

Before performing a neutralization assay to measure the presence of antibodies in serum or product, a known virus must first be grown and titrated in the test system in which the neutralization assay will be performed. For viruses prepared in cell culture, this usually involves inoculating susceptible cultures with relatively low multiplicity of infection (MOI; <1 PFU per cell) and harvesting the infected cells when about 50% to 75% cytopathic effect (CPE) is demonstrated. The virus preparation is then titrated by preparing serial multifold dilutions and inoculating replicate tubes or plate cultures with a fixed volume of the virus preparation. The endpoint of the titration is the dilution of the virus that will infect 50% of the cell cultures inoculated. This endpoint is said to contain one 50% tissue culture infective dose ($TCID_{50}$) in the volume used. If the test system involves animal lethality, the endpoint is referred as one 50% lethal dose (LD_{50}). The amount of virus used in the neutralization assay to follow is typically standardized to contain 100 $TCID_{50}$ or LD_{50}.

The test or host system used in neutralization assays is chosen on the basis of the specific virus to be tested and its ability to replicate in the system. The commonly used host systems include cell culture, embryonated chicken eggs, and mice. Cell culture is usually the preferred test system, because the viruses used in the neutralization assay usually readily replicate and produce CPE. Susceptible host cells are grown in monolayers in dishes or multiwell plate cultures. After the virus/neutralizing serum mixture is added, the cultures are overlaid with agar-containing medium to restrict spread of CPE and allow development of viral plaques. The prevention of plaque development is indicative of the presence of neutralizing antibody. Alternatively, neutralization can be performed in tube monolayer cultures or even in suspension tissue culture. Embryonated eggs may be used when the virus to be used or tested does not produce plaques in tissue culture systems. The route of inoculation and the endpoint depend on the virus.

Neutralization assays may be set up in various ways, depending on the specific virus of interest and the serum or product to be tested for neutralizing activity. In general, a fixed amount of infectious virus is preincubated with undiluted and serial dilutions of serum or product to be tested for neutralizing activity and separately with preimmune serum or control product; this approach is referred to as the *constant virus–varying serum* method. Following preincubation, the mixtures are separately injected or added to the test system. Reduction in infectivity between test and control serum or product is scored in various ways, depending on the test system. The endpoint of the assay is generally defined as the highest dilution of the serum or product that neutralizes one-half of the initial viral inoculum, as calculated by Reed-Muench or the Spearman-Kärber method.

The titer of neutralizing antibody in the test serum or product is the reciprocal of the highest dilution that completely inhibits CPE or other virus effect in the test system. This dilution is said to contain 1 neutralizing antibody unit per unit volume used in the titration. When a serum or product known to contain neutralizing antibody is used in an assay to determine the identity of an unknown virus, 20 neutralizing antibody units in a fixed volume are generally used in the assay. Positive and negative control sera must give expected reactivity in the assay.

HEMAGGLUTINATION INHIBITION (HAI)

A number of enveloped viruses, including the influenza and parainfluenza viruses, acquire protein receptors capable of binding RBCs (hemagglutinins) of various animal species on their surface as they bud through infected cell plasma membranes during viral maturation. In addition, some nonenveloped viruses such as adenoviruses and certain enteroviruses have hemagglutinin proteins in their outer capsid. This property allows for detection of a specific virus in a sample if a known specific antibody to the virus is available. Alternatively, the presence of antibody specific to the virus can be detected and quantitated by its ability to inhibit hemagglutination. This is the principle of the hemagglutination inhibition (HAI) test.

The HAI test for antibody is performed by making serial dilutions of the specimen to be tested and mixing the dilutions with a fixed amount of the virus or specific viral hemagglutinin protein in a tube or microtitration plate format. Indicator RBCs from the appropriate animal species are added, the suspension is mixed, and the tubes or plates are allowed to stand for a predetermined period. If specific antibody is present, the virus will bind and the RBCs will not agglutinate; they will settle to the bottom of the tube or plate and form an RBC "button". If specific antibody is absent, the RBCs will be agglutinated by the virus and form a diffuse film. The titer of the serum or product is the reciprocal of the dilution that completely inhibits agglutination.

HAI is very useful for subtyping influenza virus isolates. A number of factors contribute to the potential variability of the HAI test. Certain serum samples and products may contain nonspecific inhibitors of RBC agglutinins, which may yield false-positive results. A number of procedures have been developed to remove such inhibitors, including adsorption and heat inactivation procedures. Specimens may also contain RBC agglutinins other than specific antibody, and these may contribute to false-negative results. Appropriate preparation and titration of reagents, including RBCs and viral hemagglutinin stocks and suspensions, is critical. In addition, controls for nonspecific agglutination or inhibitors of agglutination must be included in every assay.

WESTERN BLOT (OR IMMUNOBLOT) ASSAY FOR ANTIBODY DETECTION

The immunoblot, or Western blot, assay is a technique for the simultaneous detection of antibodies to various protein antigens of a given virus. The term *recombinant immunoblot assay* (RIBA) is applicable when the starting protein mixtures are recombinant proteins obtained from prokaryotic or eukaryotic expression systems instead of crude or partially purified virus

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from infected cells. The method is often used diagnostically as a supplementary or confirmatory test in situations where an initial assay for antibody lacks sufficient specificity or is known to be prone to false-positive results. This is especially important when the test is being used to diagnose an infection of clinical significance such as HIV or HCV infection.

A number of commercial immunoblot kits are available, particularly for viruses such as HIV and HCV; several have regulatory approval for diagnostic use. Alternatively, viral antigen preparations may be produced in-house or purchased, along with other reagents required for the assays. Careful control and/or sourcing of these reagents are critical to ensuring that compliance requirements are maintained.

For selected viral agents, there are generally accepted interpretive standards for the analysis of reactivity or positive results in an immunoblot assay. However, the presence of nonspecific bands may be due to antibody reactivity to cellular protein antigens caused by autoimmune diseases and/or the use of crude virus-infected cell proteins as antigen in the assay. Indeterminate reactions may also occur if only a limited number of specific antibody bands are observed.

Appropriate positive and negative control sera must be included in each assay and reactivity must be scored for both the presence and the intensity of expected protein bands.

Application of the Antibody Detection Methods to Specific Viruses

Human blood-borne pathogens that may be present in infectious form in human donated blood used directly in the production of biological products are a concern because they may present a risk of transmission to others. Testing for virus-specific antibodies in donated blood serves as a screening procedure for the elimination of suspect units. Alternatively, the viruses may represent important agents for which human vaccines have been or are being developed. Thus the ability to detect virus-specific antibodies in an immunized individual or animal model may be important for demonstrating the efficacy of the vaccine. Currently, in the United States, a number of FDA-approved screening or definitive tests may be conducted on donated units of blood for evidence of the presence of agents of infectious diseases, including hepatitis B and C viruses, human immunodeficiency virus, and West Nile virus. In addition, plasma sent for fractionation before production of plasma-derived products is required to be tested for hepatitis A virus (HAV) and human parvovirus B-19.

GLOSSARY

Acceptance Criteria: Anticipated results, which may be numerical limits, ranges, or other characterization for the tests described. They establish the standards to which a drug substance or drug product should conform in order to be considered acceptable for its intended use.

Adventitious Agent: Acquired accidental contaminant in a cell line such as viruses and toxins; the agent is often infectious.

Amplicon: A segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers.

Antibody: An infection-fighting protein molecule that binds, neutralizes, and helps destroy foreign microorganisms or toxins. Also known as immunoglobulins, antibodies are produced by the immune system in response to antigens.

Antigen: Any agent that induces the production of an antibody and reacts specifically with it.

Assay Validation: A formal, archived demonstration of the analytical performance of an assay that provides justification for use of the assay for an intended purpose and a range of acceptable potency values.

Bioassay: Analytical method that uses living animals, cells, tissues, or organisms as test subjects.

Biologics: Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations —Code of Federal Regulations, Title 21 Parts 600–680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration. [Definition from *Biologics* ⟨1041⟩, *USP–NF* vol. 30 (2007), p. 414.]

Biotechnology-Derived Product: Macromolecular article derived from biotechnology processes such as recombinant DNA (rDNA) technology, hybridoma technology, and the like.

Bulk Harvest: See *Unprocessed Bulk Harvest.*

Capsid: The outer protein shell of a virus particle.

Cell Bank: A defined population of cells, such as an immortalized cell line, grown by a defined process and cryopreserved in a defined process and within a defined passage number range. The assumption is that each vial from a cell bank is comparable and that when thawed and added to a manufacturing vessel (or an analytical assay), it will perform in a consistent way.

Chaotropic: A reagent that causes molecular structure to be disrupted; in particular, those formed by noncovalent forces such as hydrogen bonding, van der Waals interactions, and the hydrophobic effect.

Complement: A group of proteins in the blood that work in concert with other immune system proteins and cells (such as antibodies) in attaching foreign substances.

cDNA: Complementary DNA. Two strands of nucleic acid that can hybridize by specific base pairing between the nucleotides.

Confluency: Refers to the point when 100% of the surface area of the vessel is covered in cells.

Cryopreservative: Reagent used to keep a cell alive in deep-frozen condition (usually in liquid nitrogen).

Cytopathic: Damaging to cells, causing them to exhibit signs of disease or cell death.

ELISA: Enzyme-linked immunosorbent assay. A biochemical technique used to detect the presence of an antibody or an antigen in a sample.

Endpoint Assay: An analytical method that measures the amount of accumulated product at the end of the assay.

Epitope: A molecular region on the surface of an antigen that is recognized by an antibody and can combine with the specific antibody produced by such a response; also called a determinant or an antigenic determinant.

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Glycoprotein: Protein that contains sugar side chains added as a posttranslational process; the presence of sugar side chains often affects activity, antigenicity, and in vivo stability.

Host Cell Tropism: The range of susceptible cells that a particular microorganism can infect.

ICH: The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Limit of Detection (LOD): The lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually 2 or 3.

Mycoplasma: Parasitic microorganism that infects mammalian cells, possessing some characteristics of both bacteria and viruses. Prokaryotic microorganisms belong to the family *Mycoplasmataceae,* with no cell walls. They may grow attached or close to cell surfaces in the cytoplasm and subtly change the properties of the cells.

Passage: An operational procedure used to feed cultured cells, usually by providing fresh medium and dilution of cells in a new culture vessel. The number of such operations is referred to as the passage number. It is not the same as cell generation number, which is strictly related to cell doubling time.

qPCR: Quantitative polymerase chain reaction. A modification of the polymerase chain reaction used to measure the quantity of DNA, complementary DNA, or ribonucleic acid present in a sample. Like other forms of polymerase chain reaction, the process is used to amplify DNA samples via the enzyme DNA polymerase.

Raw Materials: All components used to manufacture a drug substance or drug product; regulated by 21 CFR 211.

RT-PCR: Reverse transcriptase polymerase chain reaction. A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

Serotype: The kind of microorganism as characterized by testing for recognizable antigens on the surface of cells of the microorganism.

Spiking: Adding a known amount of analyte from a laboratory standard acting as a tracer to check a method for recovery or accuracy.

Syncytium: A multinucleated mass of cytoplasm that is not separated into individual cells.

System Suitability: The checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution, and reproducibility are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability *sample,* which is a mixture of main components and expected by-products.

$TCID_{50}$: 50% tissue culture infective dose. The level of dilution of a virus at which half of a series of laboratory wells contain active, growing virus.

Unprocessed Bulk Harvest: The pooled harvests of cell culture fluids that constitute a homogeneous mixture for manufacture into a unique lot of product.

APPENDIX

Relevant Regulatory References

1. CBER (Center for Biologics Evaluation and Research), U.S. Food and Drug Administration. Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals. 1993.
2. CBER (Center for Biologics Evaluation and Research), U.S. Food and Drug Administration. Letter to manufacturers of biological products. 2000.
3. 9 CFR 113.53.
4. 9 CFR 113.47.
5. 9 CFR 113.52.
6. 21 CFR 211.
7. 21 CFR 600.3.
8. Federal Food, Drug, and Cosmetic Act (FD&C Act), sections 201 (g) and (h).
9. Guidance on Virus Validation Studies (CPMP/BWP/268/95 EMEA, Feb. 1996).
10. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Q5A (R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. 1997.
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⟨1238⟩ VACCINES FOR HUMAN USE—BACTERIAL VACCINES

INTRODUCTION

An overview of vaccines for human use is presented in *Vaccines for Human Use—General Considerations* ⟨1235⟩. Bacterial vaccines can be derived from whole cells, either killed or attenuated in their ability to cause disease, or from some component(s) of the intact cell that are important for virulence or damage to the host. Another subset of bacterial vaccines, derived from

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toxins, is the toxoids. Bacterial vaccine products can be mixtures of components from different species, from different strains or different serotypes of the same species, or from different components from cells of the same species.

The simplest bacterial vaccines consist of the purified cell-surface capsular polysaccharides (CPS) from organisms such as *Salmonella enterica* serovar Typhi, various meningococcal serogroups, or pneumococcal serotypes that cause meningitis, otitis media, acute respiratory infections, and pneumonia. Although the typhoid vaccine consists of a single polysaccharide, the meningococcal vaccines contain as many as four serogroup-specific CPS, and the pneumococcal vaccine contains 23 serotypes.

The immunological response to meningococcal and pneumococcal polysaccharides, and to the capsular polysaccharide from *Haemophilus influenzae* type b (Hib), is improved by covalent attachment of the CPS or an oligosaccharide derived from it to a suitable carrier protein. The immunological response to these glycoconjugate vaccines is elicited via immunologic pathways different from those induced by purified polysaccharides, creates a T-cell–dependent response, and establishes immunological memory. The carrier proteins are typically bacterial toxoids or bacterial outer membrane protein vesicles but may also be from other sources. For these products, anti-CPS antibodies appear to be sufficient to protect against disease, although the glycoconjugate vaccines also may reduce carriage of the organisms in the nasopharynx. Due to the complexity of their manufacturing processes, glycoconjugate vaccine products tend to contain fewer serotype or serogroup components than do the related purified polysaccharide vaccines.

Many bacterial pathogens, including those that cause diphtheria and tetanus, produce toxins that kill tissue. Immunological neutralization of these toxins is sufficient to prevent disease. These subunit vaccines consist of chemically detoxified toxins (toxoids) purified from culture supernatant and are capable of eliciting neutralizing antibodies against the native toxin.Other types of purified subunit and purification processes may be developed.

Although earlier pertussis vaccines consisted of myriad chemically inactivated whole-cell and toxin components, current acellular products contain various combinations of specific purified proteins, sometimes toxoided (e.g., fimbriae and other cell-surface protein components). Compared to older products, these vaccines apparently produce protection by a different mode of action but have a lower incidence of adverse events. A combination of diphtheria and tetanus toxoids and an acellular pertussis vaccine form the core components of many polyvalent pediatric and adult combination vaccines. To these may also be added an Hib glycoconjugate, hepatitis B, and/or inactivated poliovirus immunogens.

Live attenuated bacterial vaccines are currently limited to Bacillus Calmette-Guérin (BCG), which protects against tuberculosis when administered through the skin, and the *S. typhi* Ty21a construct, which is an oral vaccine against typhoid fever.

The immune response against these bacterial polysaccharide and protein antigens can be increased by inclusion of adjuvants. The primary adjuvant licensed in the United States is based on aluminum salts such as aluminum hydroxide and aluminum phosphate, although development and characterization of new adjuvants is an active area of research.

RAW MATERIALS

Raw materials can directly affect the identity, strength, purity, and quality of bacterial vaccines. A consistent manufacturing process critically depends on use of consistent raw materials (e.g., during seed banking, fermentation, harvest, purification, and formulation; see *Vaccines for Human Use—General Considerations* ⟨1235⟩). Raw materials for bacterial growth media typically consist of both well-defined chemical entities (e.g., amino acids, carbohydrates, vitamins, minerals) and more complex components (e.g., protein hydrolysates, yeast extracts, peptones). Manufacturers should consider the source of each of these raw materials to ensure that they come from reliable vendors who adhere to cGMP quality standards and can assure a long-term supply. Manufacturers should communicate with raw material vendors in order to avoid any changes in the sourcing or manufacture of components and to avoid supply shortages. Without such communications, the consistency of the fermentation process and the supply of the vaccine can be adversely affected. Consistent raw materials are particularly critical for more complex fermentation components such as yeast extract or peptones for which changes may be difficult to detect but are likely to have a direct effect on fermentation.

Accurate records of the composition and source of the culture medium used in seed banking and routine fermentation should be maintained and also document release criteria for raw materials or components. Manufacturers should determine if any of their raw materials are derived from animal origin. If additives from animal sources are added to the culture medium, they should be certified to be free from contaminants and adventitious agents such as those that cause bovine spongiform encephalopathy or transmissible spongiform encephalopathy. Vendors/manufacturers should provide information about the identity and source of additives and should test for adventitious agents. Use of antibiotics should be minimal or should be avoided to ensure that no unwanted antibiotics are included in the drug product, unless they are intentionally used in manufacturing (e.g., as selective markers).

As manufacturers scale up fermentation to pilot production (i.e., within tenfold of final manufacturing scale), they also should ensure, to the extent possible, the availability of multiple sources for all raw materials. This will ensure that supply or business instabilities at one vendor do not become the limiting factor in vaccine manufacture.

CELL BANKS

Source and History

The source of cells used in cell banks should be documented. The original isolate should include, when possible, the age, sex, and species of the donor; the donor's medical history; and, if available, culture history including methods used for the isolation of the substrate bacteria.

The source of cells from which the strain was derived is to be stated, and relevant references from the scientific literature should be cited. The source should generate a sufficient amount of antigen(s) to meet the medical need. Information obtained directly from the source laboratory is preferred. When this is not available, literature references can be used to provide bacterial classification (i.e., genus, species, and strain designation) and specific phenotypic and/or genotypic trait. For

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microbial-expression systems such as *E. coli* or *S. pneumoniae*, the manufacturer should describe the method used to prepare the DNA coding for the protein, including the cell and origin of the source nucleic acid. All propagations carried out with the original isolate should be documented and should include, as applicable, the method used for subculture, any use of animal-derived material, record of subcultivations, and storage conditions. Constituents of the culture medium must be described, in particular, materials of human or animal origin such as serum, enzymes, hydrolysates, or other living cells.

For microbial-expression systems, the steps in the assembly of the expression construct must be described in detail. This description should include the source and function of the component parts of the expression construct (e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether or not the protein is synthesized as a fusion protein). Manufacturers should provide restriction endonuclease digestion maps that illustrate the sites used in preparing the expression construct and sites used in identification of DNA fragments.

A complete nucleotide sequence analysis of the expression construct's coding region for the protein of interest should be performed. The sequence analysis should be provided and should include a complete annotation designating all of the important sequence features. The copy number and physical state of the expression construct should be determined.

Cell Bank Lineage and Genealogy

A flow chart can be used to demonstrate the preparation of the cell bank lineage from the original source, through preliminary cell banks (or process development cell banks, as applicable), to the Master Cell Bank (MCB) and production Working Cell Banks (WCB).

Manufacturers should describe their strategy for providing a continued supply of cells from their cell bank(s), including the lot size and anticipated use rate of the cell bank(s) for production, the expected intervals between generation of new cell bank(s), and the criteria for qualification of cell bank(s). If multiple WCBs were used for clinical trials, process validation, or commercial supplies, flow charts can help illustrate the common source (i.e., MCB) from which the WCB were derived.

Once an MCB is produced, a cell bank system should be generated to prevent unwanted drift that might ensue from repeated subcultures or multiple generations. The system should ensure that an adequate supply of equivalent cells exists over the entire life span of the product. Ordinarily, the cell bank system consists of two tiers: an MCB and a series of WCB derived from the MCB. When additional tiers of WCBs are prepared, manufacturers should clearly identify the generation that will be used for WCB.

Cell Bank Manufacture

Generally, the MCB is made from a preliminary cell bank derived from the original source or directly from an initial clone. Manufacturers generally prepare cells for banking by expanding cultures in a progressively greater number of vessels or in larger vessels until a pool of cells is obtained. If manufacturers use more than one vessel, they can ensure the uniform composition of the contents by combining the cells from all of the culture vessels.

For microbial-expression systems, a single host cell that contains the expression construct is propagated to generate the MCB. Manufacturers should document the cell cloning history and method of transferring the expression construct into the host cell. They also should completely describe methods and criteria used to amplify the expression construct and to select the cell clone for production.

The process for WCB used in clinical trials and for commercial supply should be similar to the MCB process. A WCB is derived from one or more containers of the MCB and is typically used to directly provide cells for the manufacturing process. Additional WCB are generated from the MCB as needed.

Preferably the MCB and WCB should be prepared in a similar manner, but the MCB and WCB may differ in certain respects (e.g., culture components and culture conditions). Similarly, the culture conditions used to prepare the MCB and WCB may differ from those used for the production process or between clinical trial materials or commercial supply. The preparation procedures for all cell culture processes must be described, and details of process changes must be documented. Comparability of product quality must be demonstrated when process changes occur between WCBs.

Cell banks should be made under cGMP because they are expected to last for the lifetime of the product. The facility should be operated to minimize the chance of microbial contamination and have in place procedures to prevent cross-contamination with other materials. Critical equipment used in the preparation of cell banks should be qualified. Manufacturers should establish the cell bank in a suitably controlled environment to protect both the cell bank and personnel handling it. During the establishment of the cell bank, no other living infectious material (e.g., viruses, cell lines, or cell strains) can be handled simultaneously in the same area.

Cell Bank Validation

The cell banking process should be considered a unit operation and should be validated. The process begins with the MCB vial and the cell bank process validated for preparing WCB. The suitability of WCB for intended use should be further demonstrated by the consistency and quality of successive product batches. Qualified banks should be used for process validation of fermentation, drug substance, etc. If this is not possible, then manufacturers should perform a small-scale demonstration of the appropriateness of the cell bank. The basic principles of process validation apply, including use of validated analytical methods and stability evaluation.

Manufacturers should describe the methods used to preserve cell banks, including the cryoprotectant and media used. Storage containers (e.g., vials, ampules, and other appropriate vessels) and closure systems should be described. Container–closure systems should incorporate materials and designs that withstand storage and retrieval without breakage or leakage and are physically and chemically compatible with the stored material.

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Cell Bank Testing

A newly prepared cell bank (MCB or WCB) should be evaluated by a series of appropriate release and characterization tests on an aliquot of the cell bank or on cultures derived from it, as appropriate. The amount of testing required for an MCB may influence that required for subsequent WCBs, and the extent of testing both may influence the testing needed for production cell cultures. Manufacturers should evaluate all cell banks, including bacterial cultures or recombinant bacterial expression systems, for identity, culture purity, and viability. Additionally, manufacturers should evaluate the genetic stability and consistent productivity of all cell lines.

To confirm identity, manufacturers should perform appropriate tests to determine that the banked cells are what they are represented to be. Either phenotypic or genotypic characteristics can be used in identity testing to classify bacterial strains to species level, and when applicable, supplementary serological tests can be performed. For most microbial cells and transfected cells, analysis of growth on selective media is usually adequate to confirm host cell identity. Where a variety of strains can be used, biological characterization methods such as phage typing should be considered as supplementary tests. Expression of the desired product is also considered adequate to confirm the identity of the microbial expression system.

It must also be demonstrated that cell banks are biologically pure (i.e., free from adventitious microbial agents). Testing for adventitious agents should include tests for bacteria, fungi, mycoplasmas and viruses, as applicable.

Additionally, all cell banks should be tested to confirm the viability of the cells. Viable cell counts or growth tests should be performed to demonstrate that the cell culture has sufficient viability and is suitable for its subsequent intended use.

Evaluation of genetic stability and persistence of productivity is a reflection of how many doublings the cells can tolerate without compromising their genetic integrity (e.g., plasmid retention) and productivity (e.g., mass of product per cell). Such testing is critical to ensure that the cell line performs reliably in the full course of the production process from the initial MCB stage through the longest production intended. As part of this evaluation, manufacturers should document the number of passages from the original source, the number of subcultivations from the original source to the MCB, from the MCB to the WCB, and from the WCB to the final bulk. The earliest and latest culture states (e.g., MCB and end production) should be evaluated to ensure that the desired characteristics persist. Such a demonstration of cell line stability is commonly performed once for each product marketing application.

Characterization tests may be useful for demonstrating that the cell bank is composed of cells with the intended phenotypic/genotypic characteristics. Such tests can include cellular and colony morphology (i.e., use of selective and/or differential media), biochemical profiles (enzymatic activity or substrate utilization), immunological identity, characteristic growth, and antibiotic susceptibility.

Additionally, for recombinant bacterial expression cell lines (e.g., *E. coli*) molecular characterization testing can include DNA sequencing of the target gene sequence along with the flanking regions, expression construct retention, and plasmid copy number. Analysis of the expression construct at the nucleic acid level should be performed with consideration that this verifies only the coding sequence of a recombinant gene. Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for insertions or deletions and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells that retain the expression construct should be determined under selected and nonselected growth conditions. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies.

Much of this testing should be conducted on the MCB if possible, which will preclude the need to repeat much of the testing on each WCB or production lot, although sometimes redundant testing (on both MCB and WCB) may be desirable.

Limited identity testing is generally performed on each WCB if extensive identity testing was performed on the MCB. For recombinant products, the identity of the WCB should be assessed by restriction endonuclease mapping of the expression construct for copy number and for insertions or deletions. In addition, where appropriate, the WCB should be identified by phenotypic characterization (e.g., auxotrophy, antibiotic resistance).

For each lot of WCB derived from the MCB, manufacturers should routinely test for contaminants that may have been introduced from the culture medium during preparation. Purity tests like those performed on the MCB to test for adventitious agents may be performed on the WCB.

Characteristics of the recombinant protein product can also be applied (see below) as another means of defining the ultimate output of the cell line.

In the event that a new MCB is needed, the testing performed on a new MCB should be the same as that performed on the original MCB unless justified. If a new MCB is to be generated by expression construct transfer into host cells followed by clonal selection, then acceptance criteria for both the new clone and the protein produced by the clone should be described and justified.

Cell Bank Storage

In both MCB and WCB of the same product, similar containers (such as cryovials) are generally used and are treated identically during storage.

The location, identity, and detailed inventory of individual ampules of cells should be thoroughly documented with procedures that allow the cell bank containers to be traced. Labeling should clearly indicate the biological name of the components, unique container number, lot or batch number if applicable, and the type of bank (such as MCB or WCB). The label must withstand storage and retrieval without loss of integrity or information.

Cell banks should be established, stored, and used in a way that minimizes the risk of contamination or cross-contamination by other cell types that may be present in storage. Once issued, banked materials cannot be returned to the controlled storage area. Access to banked material must be controlled by a strict inventory-control system with limited access by authorized individuals only.

Bacterial cell banks should be stored in either the liquid or vapor phase of liquid nitrogen or in mechanical freezers (generally ≤−60°) . Storage conditions (generally ≤−60°) may be acceptable when supported by data that demonstrate that a minimum level of cell viability is maintained and is adequate for production use. Storage temperature and other critical storage conditions

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should be maintained within validated limits. Temperatures must be continuously monitored and recorded, preferably on an alarm system. Shipping containers used to transport cryopreserved cell banks to offsite storage facilities or manufacturing facilities must be validated, and shipping qualification must be performed before use.

Because of more frequent usage of WCBs and to protect the MCB, the WCB should be stored separately from the MCB. Cell banks may also be stored in two or more widely separate areas within the production facility, as well as at a distant site in order to avoid loss of the cell bank (e.g., caused by equipment malfunctions or disaster at the site). When stored in different locations, the cell banks must be stored under the same conditions.

As part of a disaster recovery plan, the manufacturer should document the steps and timeline needed to restart production of new cell banks and/or contingency plans for continued manufacturing production.

Storage Stability

MCB and WCB should be placed in a stability program. Evidence for banked cell stability under defined storage conditions usually is generated during production of clinical trial material or commercial material from the banked cells. Data from the determination of cell viability when the preserved cells are reconstituted for production of clinical trial supplies can verify that the revived cells have survived the preservation process. Data from the preparation of clinical materials are used to demonstrate that the revived cells can be used to prepare the desired product.

Enough MCB material for the lifetime of the product should be put on stability (enough WCB should be put on stability to support the lifetime of the WCB). This can be a large volume because the product lifetime can be quite long (e.g., 50 years). During the preparation of the MCB, the lot size should be large enough to allow adequate inventory to support the lifetime of the stability study as well as production for the life of the product. Time points for such a long-term study might include 0, 6, and 12 months, and then perhaps every 1 to 3 years thereafter. Typically, no expiration dating is used for cell banks because stability studies are used to confirm the suitability of the material. Greater reliance is placed on the successful (and typical) culture of the cells themselves. The proposed monitoring should be documented in pre-approved protocols. The time points can be reduced (e.g., increase the time between time points) if data indicate stability. In addition, time points can be added if sufficient material is available and the data suggest that more monitoring is needed. The stability plan depends on the use rate in manufacturing.

FERMENTATION

Production of the drug substance for a bacterial vaccine requires a fermentation process that is consistent and sufficiently productive to support commercial production. The approach to achieving this has become fairly standardized and provides a relatively high probability of success for early batch production to support a development program. It is still a significant challenge to achieve sufficient productivity to support commercial manufacture of a licensed product. Directly following any fermentation process is the harvest process, which serves as a transition step between biomass expansion and downstream process steps. For purposes of this chapter, harvest will be considered as an extension of the fermentation process.

Fermentation Starting Materials: Cell Inoculum

The cell inoculum for the fermentation process is the single most important component for establishing a reproducible fermentation process. In early development before finalizing fermentation conditions, manufacturers typically must generate an interim source of this inoculum, a Process Development Cell Bank (PDCB). The origin of the PDCB should be a clonal isolate of the original transfected or isolated strain that demonstrates suitable growth properties and produces the antigen of interest in sufficient quantity and quality for the intended purpose. The use of a clonal isolate ensures that the genetic starting point for each batch is the same and that subtle variations in process conditions will not inadvertently allow one population versus another to dominate the culture. That is, the PDCB is used for fermentation development to ensure that variations in the fermentation conditions can be interpreted without the overlay of competition between populations of transfectants.

Initial development of the fermentation process, preferably with the PDCB, typically precedes production of the MCB and WCB. Best practice is to derive these cell banks from the same clonal isolate as the PDCB in order to reduce the need for a second cycle of fermentation development when the WCB is deployed. Substitution of a WCB for the PDCB at the final stages of fermentation development is common practice, but care must be taken to constrain such experiments to optimization of the fermentation process. More detail is found in the cell banks section above.

Fermentation Hardware

The biomass production process typically begins with a small-volume inoculum in an initial fermentation volume that is 20- to 100-fold larger than the initial inoculum volume. This initial passage is often followed by one or more intermediate fermentations that expand the production volume by 20- to 100-fold at each step until the production fermentation volume (typically 500–3000 L) is reached. Routine manufacture at these scales requires well-controlled fermentation conditions and physical facilities that meet the economic and cGMP needs for a successful product.

Bacterial fermentations have traditionally been carried out in glass, glass-lined, or passivated stainless steel fermenters that comply with cGMP requirements, particularly when using large fermenters (e.g., those with >1000 L working volume) because of containment issues with such large volumes of liquid. Traditional fermentation systems require hard-piped control systems that meet the need for clean-in-place and steam-in-place capability. The bioburden and complexity of the facility are increased if the fermentation operations must accommodate multiple product lines as well.

Smaller fermentation batches are increasingly performed in disposable bioreactors such as single-use bags with completely disposable product contact surfaces, including sensors and probes. These systems are becoming readily available, are less

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expensive, and are more flexible than fixed equipment and meet the needs of the competitive business and evolving cGMP expectations and requirements. A note of caution is warranted, though, because this disposable technology can lead to changes in the material of product contact surfaces. Such changes then require re-evaluation and sometimes revalidation of the manufacturing process for late-stage development and commercial products. Thus the reduced cleaning burden may bring an increase in the need for extractability and leachability studies.

Harvest Hardware

Harvest of the fermentation product can focus on recovery of either the wet cell mass from which the product will be extracted or the fermentation broth from which the product will be directly purified. In the former case, centrifugal separation is typically employed. Production-scale centrifuges can be either closed operations with a fixed volume of input and manual recovery of the pellet or continuous-flow operations that automatically eject and recover the clarified supernatant and/or the accumulated pellet. Although centrifuges are efficient in harvesting a fermentation product, shear forces can have significant effects on the product stream (e.g., lysed cells, sheared molecules in solution). Alternatively, and particularly when the product is secreted into the solution rather than retained in the cells, membrane filtration systems may be used to clarify the product stream for subsequent purification. Tangential-flow and depth filtration systems can be effective means of recovering soluble product with less concern about shear forces.

In all cases, monitoring the processing of the fermentation output and solids removal from the liquid matrix can be simple but effective means to monitor process consistency and comparability. Off-line tools such as high performance liquid chromatography (HPLC), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), or Western blot analyses can track product integrity issues such as aggregation or proteolysis.

Process Development

A productive, robust fermentation process is the result of careful consideration of a broad collection of variables, considered alone as well as in conjunction with product design and downstream processing. Fermentation process variables include chemical inputs (e.g., carbon sources, minerals, vitamins, trace elements, antifoam, and gases), physical inputs (e.g., temperature, mixing, and pH), and biological processes (e.g., nutrient utilization rates, metabolite levels, and inductors [quantity/type/addition duration]).

Product Requirements

A critical consideration in process development is anticipating how much of the product will be needed. Too little product caused by operating at too small a scale imposes supply constraints and often postlicensure urgency to scale up the process. In contrast, too much product results in excess inventory, expiring lots, infrequent manufacturing (itself a problem), and generally poor economics. A clear market evaluation is needed before one designs the manufacturing process or commits to a process for scale-up or -down.

Process Design

If one has a reasonably defined production need and an initial estimate of product yield, one can extrapolate the scale of the fermentation from the volume (yield), production frequency, and the expected productivity. Commercial fermentation of bacterial cultures is routinely carried out in volumes as large as 3000 L, but larger volumes are also in use. A few large lots per year can be advantageous for a very robust process but may be limited by downstream process capabilities and/or the stability of the fermentation product as a production intermediate. An additional consideration may be the difficulty in generating enough lots to ensure that the fermentation process is indeed robust. Failure of a large lot carries important financial and inventory risks.

A large number of lots can impose logistical problems if turn-around time is too tight or coordination of downstream events becomes too complex. Logistics includes quality control testing, which depends on the number rather than the size of lots. Production that involves a large number of smaller lots can also require blending of multiple intermediate lots in order to produce a final drug product lot. This can cause challenges if product-related problems occur and may entail root-cause investigations. In general, appropriate fermentation sizing results in a process that has a turn-around time of less than a week, that can be accommodated with one or a few purification runs, and that results in one to several fills of final product after each purification cycle.

Early Development Considerations

During early development of a biological product, the most important fermentation considerations are an appropriate, well-defined MCB and a fermentation process that is reasonably productive, reproducible, and scalable. The latter is often underestimated when one considers the physical, chemical, and biological control of the process as process volumes change by orders of magnitude.

The mechanics of the fermentation process are an important consideration. Fermentations are typically studied in shake flask experiments or even microscale reactors that can readily accommodate many experiments conducted in parallel. Although this is attractive for initial identification of process conditions, the ultimate culture vessel should be a controlled fermenter where growth conditions can be controlled and monitored in a more rigorous and complete manner. Manufacturers should begin work in small-scale fermenters as early as possible to ensure that robust, controlled experiments can be run to refine the initial fermentation conditions.

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Fermentation harvest processes also should be scalable. Although it is possible to scale centrifugation conditions, it is a challenge to maintain equivalent centrifugal conditions, particularly in a flow-through mode. Filtration processes can usually be scaled more predictably provided the membrane manufacturer is anticipating the needs of the process development scientist.

When manufacturers define a process, they should evaluate its robustness by purposeful deviations such as changes in sources of raw materials and time and temperature limits of unit operations. Such evaluations better define the rationale for setting process limits and for knowing which are most critical to the success of the manufacturing process.

Process Monitoring

On the basis of early development process characterization data, manufacturers should be able to identify key analytical measures that, if applied to all lots, can either verify the correct progression of the process or serve as a sentinel to determine whether a specific batch is showing signs of deviating from the typical profile. In the absence of such data, an aberrant process may go unnoticed or may not be detected until testing of a process intermediate shows either an out-of-trend or out-of-specification result.

For a fermentation process, many critical variables (e.g., optical density, pH, and specific nutrient levels) can be measured online and in real time to potentially allow intervention to bring a given process back into normal range or at least to identify the point in the process at which the deviation occurred. Such data can be valuable in identifying potential process improvements. Conversely, in the absence of such data troubleshooting can be a challenging and protracted process.

Scale-up

Just as early development requires a focus on small-scale operations, scale-up becomes essential at some point to ensure that sufficiently large lots can be made to meet program needs. As these needs become increasingly complex, larger lots are essential to ensure that multiple experiments and observations can be tied to the same lot of product, which in turn is critical to understanding critical process and product variables. If proper process engineering considerations were taken into account at the smaller production scale, scale-up can usually be done in increments of ten-fold in volume with reasonable expectation that significant process performance or product changes will not be seen. This approach may require adjustments at an intermediate scale if the initial fermentation was based on too small a volume or if the final production scale is very large. Again, process monitoring data can be very helpful in evaluating the success of the scaled-up process.

If clinical development studies are performed at less than full manufacturing scale, as they usually are, manufacturers will be obliged to relate the comparability of the process performance and the product characteristics at the different scales. Analytical data can be compelling, but in their absence or in the presence of differences, manufacturers must demonstrate that scale-related differences are not clinically significant. However, the use of comparability protocols for scale changes will have to be approved by the local regulatory authority. In order to avoid fixing something that is not broken, analysts must take care to isolate differences caused by fermentation scale-up from changes caused by harvest or purification scale-up. One way to accomplish this is to compare process intermediates obtained, as possible, during the fermentation and harvest processes. As an example, online monitoring of fermentation conditions such as pH or glucose level can be used to demonstrate similarity during the time course of the fermentation. Similarly, measurements at the end of the fermentation process (e.g., final cell density, cell viability) and intermediate measurements during harvest (e.g., turbidity of clarified broth, wet cell mass in the pellet) provide useful information for evaluating the similarity or differences during scale-up.

PURIFICATION

A general overview of purification for bacterial derived vaccines is presented in *USP Vaccines for Human Use—General Considerations* ⟨1235⟩. In addition to a description of critical processing equipment, reagents, and processing steps, manufacturers should provide the rationale for the purification process chosen for component antigens recovered from the crude harvest. As with the other processes, analysts should consider the source of all raw materials and ensure that they come from reliable vendors who adhere to cGMP and can ensure a long-term supply. The cGMPs will apply to late-stage clinical supplies and commercial materials. The removal of nonproduct-related impurities (e.g., processing reagents, endotoxin, contaminating cell proteins or nucleic acids, and other residual contaminants) should be verified.

The drug substance can be one of several types of compounds: e.g., polysaccharides (wild type or modified), proteins (wild type, mutant, toxoids, or recombinant), or products of conjugation of polysaccharides and proteins, or products of conjugation of peptides and proteins.

To define and control purification processes for drug substance and drug products, the manufacturer should establish targets for process parameters and tolerances for all critical process steps including yields, activity, and purity to ensure efficacy, safety, and consistency of the final product. Requirements for pooling, if applicable, should be established. The requirements and conditions for storage of intermediates, bulks, and final containers must be established by an official stability program. The use, reuse, regeneration, and cleaning of all drug product/drug substance contact equipment (e.g., filters, chromatographic columns and resins, tanks, and process lines) should be validated. In addition, extractable/leachable studies should be performed for all product contact equipment (e.g., disposable bag systems, chromatographic column resins, and process lines).

Polysaccharide Purification

The purification steps for polysaccharides depend on the phenotype (e.g., gram negative or positive), polysaccharide presentation (e.g., membrane bound or excreted within the supernatant), and the chemical nature of the polysaccharide itself [e.g., idealized backbone linkages (glycosidic bond, phosphodiester bond), overall charge, types of charge groups, and types of side group modifications (*O*-acetyl, uronic acid, sialic acid, *N*-acetyl, pyruvate, or *O*-methyl)]. Polysaccharide harvest methods

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determine clarification and downstream purification requirements. Clarification methods depend on whether it is necessary to perform cell lysis or only to separate cell-free broth from cellular debris. Harvest techniques include centrifugation, depth filtration, tangential-flow filtration, microfiltration, sizing filtration, or a combination of techniques. The culture may be inactivated or residual contaminants removed by selective precipitation (e.g., protein denaturation) using heat or chemical treatments (e.g., salts, detergents, enzymes, or phenol). This may require cold-storage settling before clarification.

Methods for postclarification polysaccharide precipitation are used both for isolation and purification. Fractional precipitation methods are based on overall charge or cationic binding affinities of the polysaccharide (e.g., alcoholic precipitation, ion-exchange chromatography). Agents such as cationic detergents, salts, and solvents can be used to differentially precipitate charged species from uncharged molecules. Precipitation can be performed in stepwise fashion to remove residual impurities from polysaccharide or by a series of precipitations to achieve the desired purity. Polysaccharide may be contained in either the precipitate or supernatant depending on the charge and nature of the polysaccharide. Precipitation is followed by isolation steps such as centrifugation and/or filtration during which either the precipitate is discarded or resuspended in a secondary precipitating agent until the polysaccharide is recovered. Extraction with solvents such as phenol is sometimes used to remove impurities.

An alternative and additional approach to selective precipitation methods is the use of chromatographic methods. Ion-exchange chromatography (e.g., DEAE Sepharose), hydrophobic-interaction chromatography (HIC), and gel-permeation chromatography separately and in combination have been used successfully to purify polysaccharides. At neutral pH the charge on acidic polysaccharides can be used on anion exchangers to separate acidic polysaccharides from impurities. Ion exchangers can also be used to purify neutral polysaccharides in flow-through mode, binding impurities while neutral polysaccharides flow through the column. HIC can also be used to bind impurities while the polysaccharide passes in the flow-through fraction. If there are no base-labile groups, the charge on neutral and acidic polysaccharides can be modified by addition of base to ionize hydroxyls before chromatography. The basic conditions used control the level of *N*-acetylation, and the polysaccharides can be re-acetylated as needed. Borate salts can be used to enhance separation during ion-exchange chromatography. A combination of precipitation, filtration, and chromatographic procedures can also be used.

Diafiltrations, ultrafiltrations, and intermediate drying steps can be used as needed to concentrate polysaccharides while removing low molecular weight impurities or replacing processing salts and solvents. The precipitates or column fractions can be further purified using suitable methods (e.g., enzyme treatments, solvent extractions, or column chromatography) to remove impurities such as nucleic acids, proteins, and lipopolysaccharides. A preliminary side group modification can also be included in the purification process (e.g., de-*O*-acetylation, partial depyruvylation).

The final purification step can consist of buffer exchange and filtration followed by storage of purified liquid polysaccharide (frozen) or additional final precipitation and washing of the precipitate with solvent before drying followed by storage. Drying can be performed via several types of processes (e.g., drying under vacuum or in desiccators or by lyophilization). Drying of polysaccharides can be performed in desiccators (at various temperatures) and can include several steps of grinding or fluffing and return to the desiccators for further drying. Lyophilization of polysaccharides is possible with appropriate controls if the process requires retention of bound water. Some polysaccharides may require a residual amount of moisture to maintain stability over time. The polysaccharide is then stored under suitable conditions to avoid moisture uptake.

In-Process Controls

Manufacturers identify critical process steps and perform appropriate tests to monitor the purification process. Among the latter are filter integrity tests, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Bacterial Endotoxins Test* ⟨85⟩, and other suitable tests for residues of reagents (e.g., residual reagents, solvents, enzymes, or cations) used in purification. Polysaccharide size can be influenced throughout the production process from fermentation conditions to drying conditions or by the action of mechanical stirrers, impellers, or filtration devices. If molecular size is a critical quality attribute, analysts can perform an in-process test for size (e.g., high performance size-exclusion chromatography coupled with multi-angle laser light scattering [HPSEC-MALLS]) at the appropriate process steps to monitor and control polysaccharide size. In order to demonstrate process performance and reliability, manufacturers should characterize inherent residual contaminants (e.g., protein, DNA, and endotoxins). When validation studies have demonstrated removal of residual reagents, testing of purified polysaccharides can be omitted. If material must be sterile, analysts can perform *Sterility Tests* ⟨71⟩.

Protein Purification

The classes of bacterial protein vaccines include toxoids, nontoxoids (e.g., pertussis antigens), naturally occurring mutants (e.g., CRM197) as carrier proteins, and engineered recombinant products.

TOXOIDS

At the end of fermentation, toxin-containing culture medium should be separated aseptically from the bacterial mass as soon as possible or placed in a cold room until separation can be effected. The toxin content (Lf/mL) is checked by flocculation assay using the appropriate antitoxin standard to monitor production consistency (culture should contain NLT 40 Lf/mL). The toxin is purified first to remove any components that could cause adverse reactions in humans. A typical process includes depth filtration followed by 0.2-µm filtration to assist in removal of cellular debris. Following preliminary purification, the toxin is then detoxified with formaldehyde or glutaraldehyde or any suitable chemical reagent by a method that avoids both destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly on exposure to heat. Some toxoids require a single addition of formaldehyde, but others can require multiple additions. Alternatively the toxin could be detoxified and then purified or partially purified by depth filtration, detoxified by addition of an appropriate aldehyde, filtered using 0.2-µm filtration, and then pooled. The pooled toxoid solution is further purified by clarification with activated carbon, followed by multiple ammonium sulfate precipitation steps that further fractionate and concentrate the toxoid. Typical additional purification steps include concentration, diafiltration, and/or chromatography. Purification before detoxification results in a

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purer product and can be advantageous if the toxoid is to be used as the protein component of a protein–carbohydrate conjugate (because copurifying high molecular weight glycans will be removed before detoxification).

During detoxification and purification, endotoxin testing according to *Bacterial Endotoxins Test* ⟨85⟩, and formaldehyde, protein, and irreversibility testing are performed to control and ensure consistency of the purification process. If material must be sterile, *Sterility Tests* ⟨71⟩ can be performed.

PROTEINS/RECOMBINANT PROTEINS

Proteins used to make vaccines can be recombinant (in their native state or engineered to modify certain amino acids), or they can be naturally occurring mutants that have no wild-type activity yet are capable of inducing the appropriate immune response. Proteins are harvested from the fermenter and are extracted (e.g., by mechanical and chemical disruption) then purified by suitable methods, typically consisting of filtration–concentration steps (e.g., ultrafiltration, tangential-flow filtration, diafiltration, centrifugation, selective precipitation, and even direct capture using expanded-bed chromatography or big-bead technologies). The enriched protein solution can be further purified using appropriate filtration and chromatographic steps. For all equipment that contacts drug substances (e.g., chromatographic resins, membranes, disposable bag systems, or process lines), manufacturers should assess extractables and leachables. Analysts should determine column resin life for all chromatographic systems used in the purification (including number of uses, reconditioning requirements, and storage conditions).

The type of chromatography used to purify proteins depends on the physical/chemical properties of the desired protein as well as those of other molecular entities in the harvest culture. As an example, CRM_{197} can be purified using a multistep chromatographic process: Production material is first diafiltered and then is separated by ion-exchange chromatography (DEAE-Sepharose) in order to purify the target protein from other molecular entities present in the purification stream. The peak of interest is collected, and ammonium sulfate is added, followed by 0.22-µm filtration to condition the material before loading on the hydrophobic-interaction chromatography column (Phenyl Sepharose) for purification of the target protein based on its surface hydrophobicity. The peak fraction is then diluted with Water for Injection and is separated onto a ceramic hydroxyapatite column to further purify the target protein based on its surface charge. The eluted peak is then buffer exchanged into the storage buffer by ultrafiltration/diafiltration using cross-flow membrane filtration followed by 0.22-µm filtration to yield the sterile purified concentrate.

In-process control of protein purification includes monitoring specific protein content and critical process steps as well as monitoring removal of unwanted fermentation and purification components. The pH is critical for ion-exchange chromatography, and therefore pH should be monitored. For steps designed to remove endotoxin, procedures in *Bacterial Endotoxins Test* ⟨85⟩ are used to monitor column eluants. Bioburden is monitored according to *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩ after filtration and chromatography steps. If material must be sterile, the sterility test in *Sterility Tests* ⟨71⟩ should be performed.

POLYSACCHARIDE–PROTEIN CONJUGATES

Polysaccharide preparation and activation polysaccharides used in conjugation reactions vary in size from native high molecular weight polysaccharide to oligosaccharides produced by controlled depolymerization. Some polysaccharides can also be modified (e.g., de-*O*-acetylated, partially depyruvylated). Sizing/depolymerization of polysaccharides is performed in a variety of ways (e.g., acid/base catalysis, chemical oxidation/reduction, microfluidization, or mechanical treatment). Activation of polysaccharides can be performed by several different methods depending on the lability of particular epitopes under differing depolymerization conditions or the type of conjugate desired (e.g., neoglycoconjugate or lattice-type conjugate, use of a linker molecule or direct conjugation, or reductive amination). Appropriately sized and/or activated polysaccharides are purified by suitable methods that typically consist of various combinations of concentration and filtration (ultrafiltration/diafiltration) and chromatographic methods (size-exclusion chromatography, HIC).

The in-process testing performed to monitor the depolymerization and activation process depends on the process used. Typical control tests are pH monitoring and temperature monitoring of the sizing and activation reactions. The size of the polysaccharide during depolymerization can be followed by an appropriate chromatographic procedure (e.g., HPLC-SEC RI or MALLS). Testing of depolymerized polysaccharide for select functional groups (e.g., *O*-acetyl, *N*-acetyl, or pyruvyl groups) may be required and can be determined by nuclear magnetic resonance (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩). In-process testing of the activated polysaccharide depends on the activation process used. For example, if reductive amination is used to attach a linker to the depolymerized polysaccharide, the control testing would include measurement of reducing activity (e.g., available reducing sugars), polysaccharide content (e.g., for determining the loading ratio in conjugation), and total and free linker content (e.g., for determining the number of active sites for conjugation). Depending on the activation and conjugation process used (i.e., immediate conjugation after activation), consistency in degree of polysaccharide activation may also be demonstrated as part of process validation or reflected by characteristics of the final conjugate bulk. The concentration/filtration steps of the purification process are monitored for conductivity to ensure removal of salts.

CONJUGATION

The conjugation chemistry used determines the type of conjugate made (i.e., neoglycoconjugate or lattice). The conjugate is obtained by the covalent binding of activated polysaccharides to the carrier protein. Conjugates are purified by suitable methods designed to remove residual reagents used for conjugation as well as to remove unreacted polysaccharide and protein. The removal of residual reagents is confirmed by suitable tests or by validation of the purification process. Suitable tests are carried out to determine residues of reagents used during inactivation and purification. When validation studies have demonstrated removal of residual reagents, the test on conjugate polysaccharides can be omitted.

Appropriate chromatographic procedures (HIC, SEC) and/or filtration (ultrafiltration/diafiltration, tangential-flow filtration) are used to remove the unreacted polysaccharides, protein, residual chemicals, and salts that are used in conjugation or that

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are by-products of conjugation. Bioburden testing (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩) is performed before sterile filtration.

INTERMEDIATES

An intermediate or process intermediate in vaccine manufacture is the reaction product of each step in the process except the last one, which forms the final product. Examples of intermediates are bulk-purified polysaccharides, proteins, and activated polysaccharides that conjugate to protein.

Most vaccine production processes are stepwise and take more than one elementary step to complete. An intermediate is produced from raw materials at one or more process steps (e.g., bacterial growth, extraction and purification, and chemical modification), eventually resulting in the drug substance. The identification of the key intermediates, their production, and sampling for analytical tests must be defined in controlled documents (e.g., batch records, analytical protocols).

Intermediates can be stored for considerable periods of time before further processing and can be included in a formal stability program (see *Storage Stability*, above). Stability studies in normal or accelerated conditions should be performed to define maximal hold time for intermediates and when significant process changes are implemented.

From raw material to finished drug substance, testing throughout the process ensures a quality product. Testing of intermediates is a key quality control step to ensure their identity and purity. The quality attributes of the intermediate are commonly tested in conjunction with further processing, and their release testing should be considered. Standard operating procedures (SOPs) must be properly defined for the analytical control tests. Because of their critical role in the production process, some key intermediates could be included in formal release testing, in addition to the intermediates identified for in-process testing.

Examples of tests for structural characterization of carbohydrate-based intermediates include the following:

- identity and *O*-acetylation level (nuclear magnetic resonance)
- molecular size and polydispersity (SEC–UV, SEC-Refractive Index, SEC-MALLS, SEC-Fluorescence)
- saccharide content [colorimetric assays, high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), -Fluorescence]
- specific rotation, see *Optical Rotation* ⟨781⟩
- saccharide content, *O*-acetyl content (colorimetric assays)
- counterion content, e.g., Na^+ and Ca^{2+} (inductively coupled plasma–mass spectroscopy, atomic absorption).

Examples of tests for the purity of carbohydrate-based intermediates based on estimates of the product- and process-related impurities include the following:

- endotoxin content (*Bacterial Endotoxins Test* ⟨85⟩)
- proteins, nucleic acids, proteins, cetavlon (colorimetric assays)
- water content (Karl Fischer titration; see *Water Determination* ⟨921⟩)
- volatile substances (thermogravimetry)
- organic solvents, e.g. ethanol, phenol, acetone, DMSO, or ethyl acetate (gas chromatography–flame ionization detection; see *Residual Solvents* ⟨467⟩)
- bioburden (total viable aerobic count of microbial contamination; see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩)

Examples of tests for structural characterization and purity estimation of protein-based intermediates include the following:

- identity and molecular size (SDS-PAGE, Western Blot, SEC-UV, SEC-Fluorescence, SEC-MALLS, reverse phase chromatography)
- endotoxin content (see *Bacterial Endotoxins Test* ⟨85⟩)
- residual nucleic acids [colorimetric assay; see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩]
- bioburden (total viable aerobic count of microbial contamination; see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩)
- other proteins as impurities (SDS-Page, Western Blot, SEC-UV, SEC-Fluorescence, reversed-phase chromatography)

The tests previously reported for protein-based intermediates are also applicable when the protein-based product is defined as the drug substance. In addition to the examples reported above, many other methodologies can be applied for the identity and purity evaluation.

Stability tests for intermediates can include physicochemical methods (see section on *Intermediates*, above), formally included within an analytical panel for the stability study. In addition, biological and immunochemical tests [e.g., enzyme-linked immunosorbent assay (ELISA)] can be included. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided testing is performed at sufficient steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing. If intermediates must be stored and/or subsequently shipped to a different location for further processing, the stability of these materials must be demonstrated.

DRUG SUBSTANCE

The drug substance is the final bulk that contains the antigen at the desired concentration and is ready for the addition of other ingredients (e.g., diluents, bulking agents, stabilizing excipients, adjuvants, or preservatives) to produce the finished dosage formulation.

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The drug substance is the final product of the antigen manufacture process, before the formulation of the final vaccine dosage. The final bulk may be prepared aseptically or may include a sterilization step. Sampling for analytical tests for release and stability studies (see *Storage Stability*, above) must be defined in controlled documents (e.g., batch records, analytical protocols).

Drug substances can be stored for a considerable period of time before further processing, but if it is stored the drug substance must be included in a formal stability program (see *Storage Stability*, above). Stability studies in normal or accelerated conditions should be performed to define maximal hold times. A stability program is required for formal stability studies, and the studies must be executed according to a protocol that contains detailed information about types of tests, specifications, testing intervals, and time points.

Testing of the drug substance must be performed to ensure its identity and purity. All the testing must be done according to established SOPs, and all tests must have specifications (or provisional specifications, where applicable).

Examples of tests for structural characterization of carbohydrate-based products include the following:

- identity and *O*-acetylation level [nuclear magnetic resonance (NMR)]
- total and free saccharide content [HPAEC-PAD, capillary electrophoresis (CE), or colorimetric assays]
- total and free protein content (colorimetric assay, SEC with UV, RI, or fluorescence detection, CE)
- *O*-acetyl content (colorimetric assays)
- molecular size (SEC-UV, -RI, -Fluorescence, or -MALLS)

Examples of tests for the purity of carbohydrate-based drug substances based on estimating the product- and process-related impurities include the following:

- endotoxin content (*Bacterial Endotoxins Test* ⟨85⟩)
- process-related residuals not shown to be removed by process validation (e.g., cyanide, iodate, bromide, ammonium sulfate, or organic solvents)
- Bioburden (total viable aerobic count of microbial contamination; see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩)

In addition to the examples reported above, many other methodologies can be applied for identity and purity evaluation. For instance, specific impurities that must be measured are determined by negotiations between manufacturers and the national drug regulatory agency during the licensure process. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided testing is performed at sufficient steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing.

All the results must be reported in a controlled document. Stability tests can include both physicochemical methods (see stability information, above) and biological/immunochemical tests (e.g. ELISA and SBA; see *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩).

DRUG PRODUCT AND LOT RELEASE

General principles are described in *Vaccines for Human Use—General Considerations* ⟨1235⟩, which outlines the lot release procedure in accordance with 21 CFR 610.1 and 21 CFR 610.2. For products that will be used in the United States, samples and protocols containing all appropriate tests must be submitted to FDA for review and/or testing. If FDA determines that the lot meets the standards of safety, purity, and potency required for the particular vaccine, the lot is approved for release, distribution, and marketing.

Tests required for each lot-release protocol include potency, general safety, sterility, purity, identity, and constituent materials. Potency and potency-related tests are different for each bacterial vaccine. The inclusion of these tests makes each bacterial vaccine lot-release protocol unique.

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established either by physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific cultural tests, or in vivo or in vitro immunological tests. In large part, identity testing is performed to distinguish the vaccine from other materials that are manufactured at the same site (21 CFR 610.14). The same tests that establish identity may also be appropriate for defining the quantity of immunogen present in the final vial. This is especially important for carbohydrate-based vaccines that are dosed by mass and for which physicochemical measures of antigen quality are used.

Immunochemical methods, which include immunoprecipitation methods and immunoelectrophoretic methods, have been useful. Immunoprecipitation methods, flocculation and precipitation, can be carried out in solution or in a gel matrix and involve mixing the antigen with an appropriate antibody, leading to the formation of flocculating or precipitating aggregates that can be detected visually or by light scattering (light scattering or nephelometry). The ratio of reactants must be varied to optimize the detected response. In solution this can be achieved by titrating one reactant with the other, and increased sensitivity can be obtained by using antigen- or antibody-coated particles (e.g., latex) as reactants. In gel systems, a gradient is created as one or more of the reactants diffuse, creating a visible line where precipitation occurs. Immunoelectrophoresis (IE) is a qualitative technique that combines two methods: gel electrophoresis followed by immunodiffusion. Crossed IE is a modification of the IE method that is suitable both for qualitative and quantitative analysis. Visualization and characterization of immunoprecipitation lines can be performed by selective or nonselective stains, fluorescence, enzyme or isotope labeling, or other relevant techniques. Selective staining methods are usually performed for characterization of nonprotein substances in the precipitates. In translucent gels, such as agar or agarose, the precipitation line becomes clearly visible in the gel provided that the concentration of each of the reactants is appropriate.

Where multiple active components are present as a result of copurification (e.g., certain acellular pertussis vaccines), the manufacturer must demonstrate that the composition of the product is consistent between batches, unless this has been validated during the development of the manufacturing process.

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For certain vaccines, notably those that use purified polysaccharide immunogens, identity and immunogen quantity can be demonstrated using one or more chemical and physicochemical approaches such as colorimetric determinations of different groups of sugar residues expected to be present, chromatography, or high-field nuclear magnetic resonance spectroscopy.

A number of classical colorimetric assays for quantification of various classes of sugar have been used to define the composition of polysaccharides used as vaccines, including the orcinol assay for ribose, phosphorus, sialic acid, uronic acids, and aminosugars. In general, these approaches have been superseded by chromatographic methods, including gas chromatography and HPAEC, which is widely used to determine Hib PRP glycoconjugate immunogens in monovalent or combination vaccines and in meningococcal conjugate immunogens.

Immunochemical methods that have been used to quantify polysaccharide antigens include (a) rocket immuno-electrophoresis and (b) rate nephelometry. Electroimmunoassay, also called rocket immuno-electrophoresis, is a quantitative method to determine antigens that differ in charge from the antibodies. Electrophoresis of the antigen to be determined is carried out in a gel that contains a lower concentration of the corresponding antibody. Nephelometry methods have been used to quantify antigen in pneumococcal conjugate vaccines.

The consistency of the molecular size and molecular size distribution of polysaccharide- and carbohydrate-containing conjugate vaccines can be determined by gel-permeation chromatography on appropriate resins calibrated with suitable molecular weight markers or coupled to laser light-scattering equipment that indicates absolute molecular weight if a value for the refractive index increment (*dn/dc*) is known. Measurement of the molecular size of formulated conjugates may not be feasible for multivalent glycoconjugate vaccines. Integrity of the conjugate may be demonstrated by alternative, product-specific methods. Another alternative to demonstrate integrity of the glycoconjugates in the final product is measurement of molecular size as part of the stability studies at monovalent conjugate bulk prior to formulation of the multivalent vaccine.

Unless the contrary has been validated, manufacturers should demonstrate that reversion to toxicity has not occurred (and will not occur over the shelf life) for a product derived from or containing a toxoid material. This may require the use of a cell line or an in vivo test, although enzymatic approaches are being validated.

An antigenic purity test is an assay that assesses the quantity of antigen and is used for diphtheria and tetanus toxoid vaccines. The antigen content is determined by a flocculation assay.

The manufacturer should prove a high and consistent level of immunogen adsorption to any solid-phase adjuvant (such as aluminum phosphate or aluminum hydroxide) that is consistent with the release specification.

For certain vaccines such as the anthrax vaccine and toxoid vaccines, the manufacturer is required to demonstrate that the vaccine is protective against disease or death in animal models challenged with a predefined dose of the target pathogen. This generally requires definition of the animal model, route of administration, vaccine dilutions required, a means to observe effects, and a reference vaccine against which effects are compared. The data should be analyzed appropriately (see *Analysis of Biological Assays* ⟨1034⟩).

Stability-indicating assays are those used to determine the stability of the product. Of primary importance is the potency assay, although glycan degradation may be important in glycoconjugate vaccines.

Other Vaccine Components and Vaccine Properties

Aluminum compounds are the primary adjuvants used in vaccines in the United States. General chapter *Vaccines for Human Use—General Considerations* ⟨1235⟩ provides provisions of the 21 CFR 610.15 governing the use of aluminum and amounts allowed. The adjuvants widely used in bacterial vaccines include aluminum potassium sulfate (alum), aluminum phosphate, aluminum hydroxide, and combinations of these compounds. Bacterial vaccines formulated with such adjuvants are referred to as adsorbed vaccines, and this term may be included in the official name of the vaccine. Other adjuvant systems may be evaluated. Aluminum is quantitated using colorimetric, titrimetric, emission or atomic absorption spectroscopy, or inductively coupled plasma–mass spectrometry.

For regulations regarding residual manufacturing reagents, see the FDA's 1999 *Guidance for Industry: Content and Format of Chemistry, Manufacturing, and Controls Information and Establishment Description Information for a Vaccine or Related Product.* Manufacturing reagents such as formaldehyde and glutaraldehyde sometimes are used in inactivation, the toxoid-making processes, or elsewhere during manufacture and may be present in residual amounts in the final product. Limits of formaldehyde and other residuals must be minimized in accordance with the approved product license.

Common preservatives used in bacterial vaccines include thimerosal, phenol, 2-phenoxyethanol, and benzalkonium chloride. *Vaccines for Human Use—General Considerations* ⟨1235⟩ and 21 CFR 610.15 provide additional information about the minimization of thimerosal content and the production of thimerosal-free vaccines. Limits and content specifications are set for each bacterial vaccine in the product license.

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins as indicated in *Bacterial Endotoxins Test* ⟨85⟩.

Each lot of final containers of a vaccine intended for use by injection may be tested for pyrogenic substances as indicated in *Pyrogen Test* ⟨151⟩ and 21 CFR 610.14.

Each lot of dried product shall be tested for residual moisture (see *Loss on Drying* ⟨731⟩ and FDA Guideline for the Determination of Residual Moisture in Dried Biological Products. January 1990). Residual moisture should be determined for lyophilized vaccines.

A general safety test is performed on biological products intended for administration to humans with the purpose of detecting extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR 610.11.

Excipient identity and quantity, preservatives, diluents, adjuvants, extraneous protein; and cell culture-produced vaccines and antibiotics are tested according to 21 CFR 610.15 and/or appropriate guidance documents.

"Free" or unconjugated saccharide in glycoconjugate vaccines is considered undesirable and is subject to limit specifications. As an alternative to controlling free or unconjugated saccharide, integrity of the conjugates in the final product may be demonstrated via an appropriate method, said method depending on the properties of the final product (composition, adsorption, etc). A test method that measures the increase in the amount of free saccharide is a stability-indicating method.

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The methods adopted depend on separation of saccharide from conjugate and application of the methods above to quantify the unconjugated saccharide. Separation methods used include membrane separation (such as dialysis), use of hydrophobic media to specifically trap the conjugate, solvent extraction, and selective immunochemical precipitation of the conjugate using anticarrier antibodies.

The sterility of each lot of each product is conducted according to procedures described in *Sterility Tests* ⟨71⟩ and 21 CFR 610.12 for both bulk and final container material.

Information Insert (Label)

Vaccine product labeling is regulated in compliance with 21 CFR 201 and 610. Requirements are set both for container labeling and package labeling.

OTHER REQUIREMENTS

Retention samples are held by the manufacturer for at least six months after the expiration date. Enough material of each lot of each product is held for examination and testing for safety and potency (see 21 CFR 600.13).

Records are maintained concurrently with each step in the manufacture and distribution of product such that at any time successive steps of manufacture and distribution may be traced (see 21 CFR 600.12).

For storage conditions, see 21 CFR 610.50 and 53.

For shelf life/expiry date, see 21 CFR 610.50 and 53.

⟨1240⟩ VIRUS TESTING OF HUMAN PLASMA FOR FURTHER MANUFACTURE

SCOPE

The scope of this chapter is limited to the virus testing performed on human plasma for the further manufacture of pharmaceuticals, which are referred to as plasma-derived products (see *Virology Test Methods* ⟨1237⟩ for virus testing of other therapeutic products). These types of plasma include either source plasma collected by apheresis or recovered plasma obtained from whole blood collection or as a byproduct in the production of blood components. In all cases, the source material is obtained through voluntary donations. The following topics are specifically excluded from the scope of this chapter:

- Virus testing of nonhuman blood or plasma; for example, fetal bovine serum (see *Bovine Serum* ⟨1024⟩ for more information on testing this material), which may be used in the production of biological or recombinant therapeutics
- Virus testing of human-derived whole blood, blood components used for transfusion, and materials in tissue and organ banks
- Testing for nonviral organisms; for example, bacteria, fungi, and parasites (some of these topics are discussed in *Sterility Tests* ⟨71⟩), or the causative agent of transmissible spongiform encephalopathy.

This chapter introduces the virus testing that is performed on plasma used for the production of therapeutic proteins. Topics that are addressed include:

1. The rationale for implementing tests for viruses
2. The types of testing applied to plasma donations destined for further manufacture
3. The current regulatory environment for such virus testing

The chapter also includes an *Appendix* that contains pertinent regulatory guidances and supporting references.

INTRODUCTION

Human-plasma-derived products are used to treat coagulation disorders, primary immune deficiency, and congenital emphysema as well as other diseases (see *Human Plasma* ⟨1180⟩). Because these products are manufactured from pooled human plasma donations, the presence of blood-borne viral pathogens from individual plasma donations can potentially contaminate the resulting final products manufactured from a large pool of donations and thus transmit the virus to many recipients. Sufficient measures must be taken to ensure that these products are as safe as possible. In order to minimize the risk of transmission of viruses by these products, manufacturers use several strategies, which include:

- Selection and management of the donors (see also ⟨1180⟩)
- Selection and management of donations or units (see also ⟨1180⟩)
- Testing for infectious viral pathogens in plasma in the form of samples of individual or pooled donations and fractionation pools (defined for the purposes of this document as the first homogenous pool or early production intermediate suitable for testing and representative of the material to be used for product manufacturing)
- Donor-screening methods, which also include a look-back procedure for the quarantine and destruction of unused, previously donated units from an infected donor (see also ⟨1180⟩)
- Incorporation of validated virus inactivation and removal steps (pathogen-reduction steps) into the manufacturing processes (see also ⟨1180⟩)

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- Monitoring and investigating adverse events in recipients of final products, both hemovigilance and pharmacovigilance (see also ⟨1180⟩)
- Adherence to Good Manufacturing Practices at all levels of the production process as a strategy to reduce risk of virus transmission (see GAO-HEHS-98-205, 21 CFR Part 606, and WHO Technical Report Series 941 cited in the *Appendix*).

Plasma used for further manufacture can be either source plasma or recovered plasma. In the United States, licensed human plasma products are derived mainly from source plasma. Because plasma for further manufacture is obtained by pooling a large number of donations, there is a risk of viral contamination of the pool, thus resulting in a much higher potential risk of virus transmission to multiple recipients than is the case for blood for transfusion. The manufacturing process, which is used to purify and concentrate the desired protein, is not capable of completely removing the viral load, and therefore validated virus-reduction steps capable of effectively reducing transfusion-transmissible viruses in the starting material are included in the manufacturing process. A detailed discussion of virus inactivation and removal procedures for pathogen reduction can be found in the 2004 WHO Technical Report Series 924 cited in the *Appendix*.

Approaches for screening plasma for further manufacture can be categorized into two groups: donor-screening and in-process testing methods. The donor-screening method takes into account not only the plasma-derived end product but also the plasma donor. This category of testing typically is required for blood-transmissible viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV). Virus transmission is a major public safety concern, because infections with these highly pathogenic viruses typically progress to chronicity. During donor screening the objectives of the test laboratory or manufacturer are not only to identify positive units for destruction before production pooling but also to identify and notify infected donors. Donors who test positive for HCV or HIV are permanently deferred from donating both blood and plasma. Although fewer than 5% of HBV-infected adults develop persistent asymptomatic infection (i.e., a carrier state), HBV-positive donors are deferred permanently. Collection of source plasma from donors who are convalescing from HBV is sometimes permitted for further manufacturing into plasma-derived products such as Hepatitis B Immune Globulin (Human) [21 CFR 610.41(3)].

In contrast to viruses that are associated with donor screening, viruses such as hepatitis A virus (HAV) and parvovirus B19 (B19V) usually cause self-limiting infections in immunocompetent individuals, and thus manufacturers use in-process nucleic acid amplification technology (NAT) testing that results in only the removal of plasma units with high levels of virus before pooling for production. In these cases, there is no donor-management procedure and hence no requirement to inform the donor of the result. This approach focuses primarily on the product, not the donor, because some recipients of these products are susceptible to an infection that may occur if the pathogens were present in the plasma-derived product. Donor and donation-management procedures are well developed in the blood and plasma industry, and more details on these specific topics can be found in ⟨1180⟩. Other permanent or temporary donor-deferral criteria are in place to avoid donations from potentially infected donors based on the epidemiological surveillance of a country or region or donor population for transfusion-transmissible infections relevant to the safety of blood components.

Viruses that greatly affect public health, such as HIV, HBV, and HCV, are detected by serological assays that measure either a viral antigen, such as hepatitis B surface antigen (HBsAg), or an antibody, such as anti-HCV or anti-HIV-1/2 antibodies, in infected donors and associated donations. These immunoassays for detecting viral markers in plasma donations must be sensitive and able to detect a viral infection as early as possible following infection in order to identify and exclude potentially infectious donations.

There is a finite time period, known as the window period, between the infection of a donor and the time at which the test method can detect the antibody response to the virus, the viral antigens, or the viral nucleic acid. This window period varies from disease to disease as well as from person to person. The window period can be effectively "shortened" by changing from a test based on detecting antibodies to one based on detecting the virus directly, namely the viral antigen or, especially, the viral nucleic acid (see *Figure 1*), thereby interdicting donations that contain transfusion-transmissible viruses. Tests for detecting the viral nucleic acids (i.e., NAT tests) were introduced in the 1990s. NAT tests are sensitive and can considerably shorten the window period (see *Figure 1*). In a later development after B19V transmission incidents involving plasma-derived products, NAT tests were initiated to interdict high-titer donations, thereby decreasing the B19V virus load in manufacturing pools.

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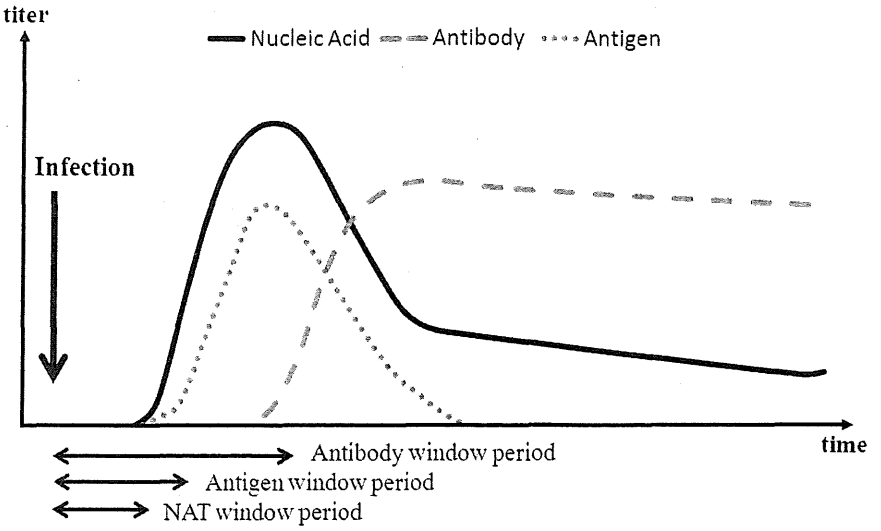

Figure 1. Dynamics of virus replication and detection of an infection.

Both source plasma and recovered plasma are tested by serological and NAT tests that are approved by competent regulatory authorities or, in the case of in-process testing, are validated to manufacturers' requirements. Plasma units found acceptable by these tests are combined into large pools, called fractionation pools, for manufacturing of plasma-derived products. The fractionation pool size can vary from several hundred donations (typically used for the production of specific immunoglobulins) to several thousand donations (used, for example, for the manufacture of albumin). Finally, the fractionation pools are retested for the target viruses. Testing of the plasma donations, at the individual or minipool level, and the fractionation pools are two of the elements that manufacturers put into place to maintain the safety margins of these products. Serology testing is performed on the individual donations. In contrast to serology tests, current NAT tests are highly sensitive and specific; therefore, manufacturers, in addition to testing individual donations, test minipools made up of equal volumes of each donation. Currently, the minipool size used for NAT testing varies from 6 to 512 donations. The high sensitivity of NAT tests also allows earlier virus detection compared to an antigen- or antibody-based test, thereby reducing the average length of the window period.

Plasma-derived products are produced from tested fractionation pools and are further manufactured by using a combination of fractionation and purification steps. These steps may have some inherent potential to remove or inactivate viruses and thus reduce viral contaminants that may have been present in the starting plasma. Nevertheless, manufacturing of plasma-derived products also includes dedicated steps designed solely to inactivate (e.g., by solvent-detergent treatment) or remove (e.g., by virus filtration) potential viral contaminants.

RATIONALE FOR VIRUS TESTING OF PLASMA FOR FURTHER MANUFACTURE

Historically, virological test methods have been used for detecting viral antigen or antibodies in clinical settings for disease diagnosis, intervention, and containment. Subsequently, these methods were adapted to screen blood and plasma donations with high sensitivity and specificity for transfusion-transmissible viruses. In order to develop a new virus screening test, scientists must know the biochemical properties of a new emerging pathogen (e.g., the nucleic acid sequence) for the development of an NAT test or the protein (e.g., antigen) for immunological tests. When implementing such a test for a given pathogen, the public health implications of positive test results and the potential for early intervention and treatment of the disease have to be considered. In addition, the availability of plasma for further manufacture and the effects of the virus on the safety of the finished product should be taken into consideration.

The emergence of a viral pathogen in the donor population could result in a considerable virus load in the plasma donations and in the resulting fractionation pools. For viruses such as HBV, HCV, HIV-1, and HIV-2 that can cause chronic diseases with potential public health effects, all donations positive for one or more of these viruses, irrespective of the virus titer, must be removed, and the donor must be informed. Some viruses such as B19V are prevalent in the population (as many as 1 in 5000 individuals may be infected during an epidemic period) and can be present at high virus titers in infected individuals. Thus, the removal of all B19V-positive donations could lead to a shortage of plasma. Instead, in-process NAT testing is done to interdict high-titer donations and thereby limit the B19V load in the manufacturing pool. The rationale for such screening is that B19V causes a self-limiting infection in most immunocompetent individuals. Following recovery, such individuals have neutralizing antibodies to B19V. Seroconversion occurs early in life, because B19V infection is common in childhood, and approximately 50% of 15-year-old adolescents have B19V antibodies. Infection of susceptible individuals continues throughout adult life, and B19V seroprevalence increases with age. The B19V neutralizing antibodies present in a plasma pool and the validated virus reduction steps included in the manufacturing process ensure that the inclusion of such donations does not compromise either the safety of the plasma-derived products or the availability of plasma for further manufacture.

In some instances it may not be necessary or feasible to test for a blood-borne virus. For example, testing of plasma for cell-associated viruses such as Human T Lymphotropic Virus (HTLV) types I and II, which present with no or with only limited virus load in plasma (but with a considerable virus load in whole blood donations) is not required. Similarly, testing for West Nile Virus (WNV), a member of the Flaviviridae family, is unnecessary because the virus load is low during the asymptomatic

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window period, the prevalence in the donor population is low (resulting in a low virus load in a plasma pool for fractionation), and WNV can be effectively inactivated by the manufacturing process as demonstrated by validation studies using relevant Flaviviridae model viruses. Therefore, WNV NAT testing for plasma (source and recovered) for further manufacture is not required. However, in the United States, the Food and Drug Administration (FDA) recommends WNV NAT testing for blood and blood components for transfusion because of the epidemiological situation and the risk of WNV transmission by blood components (see the FDA Guidance for Industry cited in the *Appendix*).

Other pathogenic viruses such as influenza viruses and severe acute respiratory syndrome coronavirus (SARS-CoV) are associated with clinical disease after a short incubation period and have a low or no virus load during the asymptomatic window period. No transmission by blood transfusion or plasma-derived products has been reported for these viruses. Furthermore, the manufacturing process for plasma-derived products has been shown to effectively inactivate influenza viruses. Therefore, NAT testing of plasma is not required for these viruses.

For a virus with a high prevalence in the donor population but without known clinical implications, no screening program, neither NAT nor serology, is required because the majority of donors would no longer be eligible to donate, thereby threatening the supply of blood and plasma and of plasma-derived products. Viruses that fall in this category are Torque Teno Virus (TTV), which is present in greater than 80% of the general population, and GB virus C (GBV-C, previously known as hepatitis G virus, HGV).

Newly emerging pathogens such as hepatitis E virus (HEV) can potentially enter the blood and plasma donor population, resulting in viral infections in recipients of blood and plasma-derived products. Monitoring the emergence of such agents is a continuous effort that involves academia, public organizations that monitor health and develop early warning systems, regulatory agencies, and industry. Currently, several epidemiological surveillance systems are in place and include hemovigilance or biovigilance to address the potential risk of emerging pathogens to the recipients of blood and plasma-derived products. This risk can be mitigated by appropriate measures, such as donor deferral because of geographic risk and risk behaviors, the testing of donations if appropriate, and the inclusion of virus-reduction steps for a wide range of enveloped and nonenveloped viruses during the manufacturing process.

APPROACHES TO TESTING

Virological screening assays are designed to detect antibodies, antigens, or nucleic acid sequences of the infectious virus via serological and NAT testing. Sensitive virological test methods are a prerequisite for the quality control of fractionation pools in order to interdict and discard infected donations before manufacturers process these donations into pools to produce plasma-derived products.

All assays used to screen blood or plasma donations should be designed for their intended use and should meet the performance requirements specified by the Clinical and Laboratory Standards Institute (CLSI) guidelines for qualitative and quantitative tests. Assays also should comply with guidance from regulators, such as the European Common technical specifications for in vitro diagnostic assays (see *Appendix*). Associated calibrators or control materials must be traceable to reference material of a higher order or to reference measurement procedures. The *Appendix* includes FDA guidance documents pertaining to the manufacture and clinical evaluation of these assays and the use of controls. U.S. and EU requirements or recommendations for tests for screening plasma for further manufacture are described in the *Regulatory Environment* section.

Serological (Immunological) Assays

Serological assays detect antibodies, antigen, or a combination of both. Antibody-detection assays usually are performed by incubating an immobilized virus antigen (virus lysate or, more common currently, virus proteins produced by recombinant protein technology) with a plasma sample. If antibodies specific to the viral protein are present in the sample, they bind to the target antigen. The virus-specific, bound antibody is in turn incubated with a labeled secondary antibody that is specific for the virus-specific, bound antibody. The label yields a signal that then is detected. Alternatively, for the measurement of a viral antigen present in a sample, immobilized antibodies specific for the viral antigen first are incubated with a plasma sample, a labeled antibody (often a monoclonal antibody) against the virus protein is added, and the mixture is incubated. For more details about these assays, see *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩.

Nucleic Acid Amplification Technology Tests

NAT is a collective term for the various methods that are used to amplify and detect the specific genomic sequences in various sample types. These methods include polymerase chain reaction (PCR), transcription-mediated amplification (TMA), and branched DNA (bDNA) and are detailed in *Nucleic Acid-Based Techniques—General* ⟨1125⟩, *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, and *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. NAT tests typically use gene-specific oligonucleotides (e.g., primers, probes), enzymatic amplification reagents (e.g., buffers, nucleotides, enzymes, cofactors), and a method that allows the detection of the resulting amplification products. Currently, NAT tests for HBV, HCV, HIV, B19V, and, in some cases, HAV, are used to screen plasma for further manufacture.

For high-throughput testing, which is desired for the testing of plasma donations used for further manufacture, NAT offers distinct advantages over serological testing. First, because of the high sensitivity of these methods, samples of plasma donations can be combined into pools (minipools) that allow simultaneous testing of multiple samples, in contrast to serological assays that are performed on individual samples. Although the operational logistics used in donation minipool testing are more complicated than those involved in testing individual donations, the minipool approach generally allows an improved turnaround time for the release of negative samples compared to the traditional nonpooling method. A reactive minipool is deconstructed to identify any positive donation(s). However, because of the dilution of virus in any given donation, minipool testing has an inherently decreased sensitivity compared to individual donation testing.

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Table 1 summarizes the potential viral load, which could be avoided by NAT testing, in a fractionation pool caused by the inclusion of a single serological-window-period donation for the five major transfusion-transmitted viruses.

Table 1

Virus	Potential Viral Load in Fractionation Pool Caused by Contamination with One Window-Period Donation (Approximate Values)[a]
HBV	8×10^5 IU
HCV	8×10^{10} IU
HIV-1	8×10^9 IU
HAV	8×10^9 IU
B19V	8×10^{14} IU

[a] Assuming one plasma donation is approximately 800 mL. References supporting these values are found in the *Appendix*.

REGULATORY ENVIRONMENT

Regulatory agencies have the goal of ensuring that plasma-derived products are safe with respect to risk from blood-borne pathogens. In addition to regulating the final products, regulators also oversee the assays used to test for infectious agents and set policies about how those tests will be used. Although regulatory agencies in the United States and Europe share the common goal of safety, they use different legal structures and strategies. In general, the hierarchy of regulatory documents is similar. Both start with laws that set the definitive requirements for donor selection and plasma screening.

In the United States, the primary laws that regulate plasma-derived products and the assays that test their safety are the Public Health Service (PHS) Act and the Federal Food, Drug, and Cosmetic (FD&C) Act. The PHS Act addresses biologics and communicable disease controls, and the FD&C Act addresses drugs and medical devices. Donor-screening tests are licensed under the PHS Act rather than being cleared or approved under the medical device provisions of the FD&C Act. Testing requirements for communicable disease agents, including viral pathogens such as HBV, HCV, and HIV, are required under 21 CFR, including not only test requirements (21 CFR 610.40) but also donor deferral (21 CFR 610.41) and look-back requirements (21 CFR 610.46 through 610.48). If a plasma or blood donation is reactive in one of the screening tests, especially in the donor-screening tests for HBV, HCV, or HIV, supplementary or confirmation tests should be conducted to clarify whether the donor is infected [21 CFR 610(b)]. The donor must be informed (21 CFR 630.6) and should be excluded from donating blood or plasma (temporarily or permanently according to 21 CFR 630.6), and manufacturers should initiate a look-back procedure (21 CFR 610.40–48). For HIV and HCV, the procedure includes not only the quarantine and destruction of unused, previously donated units from an infected donor, but also the further testing of the donor and notification of the recipients of the blood and blood components (21 CFR 610.47–48). The look-back period can be as long as 1 year (21 CFR 610.46–48).

The responsibility for legislation of the European Union (EU) is shared between the EU and the European Member States. The European Commission (EC) is responsible for the regulation of the common European market and thus is responsible for medicinal products for human use, whereas the Member States are responsible for health care, which includes the supply of hospitals with blood components such as plasma and cellular components for transfusion. Because the EC is responsible for the regulation of medicinal products derived from human blood or plasma, it is, as a consequence, also responsible for the regulation of plasma for further manufacture. The laws of the EU are found in regulations and directives from the EC and in the binding monographs of the *European Pharmacopoeia* (*Ph. Eur.*).

Additional tests and specifications for plasma for further manufacture have been developed as part of the Plasma Product Therapeutics Association (PPTA) Voluntary Standards Program. The PPTA Quality Standard for Excellence, Assurance, and Leadership (QSEAL) includes additional routine testing of blood and plasma donations or plasma pools for HCV RNA, HIV RNA, HBV DNA, HAV RNA, and B19V DNA. Companies certified by PPTA under the QSEAL program have implemented this testing.

Testing of Plasma for Further Manufacture

In the United States, all virus tests intended for donor screening, such as HBV, HCV, and HIV, are regulated as biologics and are subject to clinical validation and licensure by FDA's Center for Biologics Evaluation and Research (CBER). Clinical specificity must be evaluated and demonstrated with healthy donors and follow-up testing when applicable, and clinical sensitivity should be evaluated and demonstrated with high-risk donors and follow-up testing. Use of reference panels (from FDA or a designated source) is needed for release of each lot of kits intended for market distribution. In-process tests such as NAT testing for HAV or B19V do not require clinical trials to demonstrate assay effectiveness. However, the manufacturers of plasma-derived products should perform preclinical validation and should submit data for review and approval by CBER as analytical procedures for plasma-derived products.

In the European Union, donation screening tests are regulated as medical devices. Specifically, Directive 98/79/EC outlines requirements for the approval of tests or test kits, which require the CE mark before marketing and use for testing. The CE mark confirms that the test or the test kit meets specified quality criteria. Screening tests for manufacturing pools must be validated by the end user following specific guidelines. As in the United States, in-process tests are not licensed, and the end user is responsible for validating the test.

In Europe, in addition to virus screening of plasma pools by the manufacturers of plasma-derived products, screening for defined viruses is part of the official batch-release procedure. The EC and the Council of Europe agreed in May 1994 to create a network of Official Medicines Control Laboratories (OMCLs). The OMCLs perform tests on each batch of plasma-derived medicinal product, including virus testing of the fractionation pool used to produce the batch. All required tests are performed

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and documented in the European Batch Release Certificate that is accepted by each Member State as the basis for placing the product on the market. In order to comply with the sensitivity limits set for NAT testing, minipools of various sizes (6–512 donations) are tested by the manufacturer or by the blood donation centers where the collection and testing of blood or plasma is performed. Only donations that meet the requirements are used for pooling.

Serological Tests

FDA REQUIREMENTS OR RECOMMENDATIONS

An individual donation of source plasma or recovered plasma derived from whole blood must be tested for HBsAg, anti-HIV-1, anti-HIV-2, and anti-HCV, but not for anti-HBc, anti-HTLV-I, and anti-HTLV-II by FDA-licensed serological tests intended for donor screening (*Table 2*). A reactive donation must be further tested by a supplemental (i.e., additional, more specific) test that has been approved for such use. Even with the implementation of corresponding NAT tests, serological testing of each donation still must be performed.

Currently, FDA recommends using licensed donor-screening kits that are capable of detecting anti-HBsAg, the antibody capable of neutralizing HBV, at 0.5 ng/mL or less. Whole blood sometimes is tested for anti-HBc, but because anti-HBsAg often occurs with anti-HBc, plasma for further manufacture is not required to be tested for anti-HBc. Therefore, although recovered plasma is derived from whole-blood donations that might have been tested for anti-HBc, it can be shipped for further manufacturing regardless of the test results.

FDA first recommended standardized anti-HIV-1 donor-screen tests in 1989 in a draft Points to Consider document that described test kit manufacture and the preclinical and clinical studies needed for licensure, and this recommendation generally can be applied to other serological tests. Since the availability in 1991–1992 of licensed serological kits for simultaneous detection of antibodies to HIV-1 and HIV-2, FDA further recommends the use of either a licensed combined test or two separate licensed tests for donor screening.

FDA licensed an anti-HCV test containing multiple recombinant antigens in 1992, and a subsequent guidance recommended that all donations for blood and blood components intended for transfusion and source plasma intended for further manufacture be screened by an FDA-licensed test for anti-HCV.

EU REQUIREMENTS

Requirements for collection of blood and plasma, for selection of donors, and for testing of donations in Europe were released in 2003. Directives 2002/98/EC and 2003/63/EC contain donor-selection criteria and testing requirements for blood and plasma independent of its use. The requirements of the directives are standards for plasma for further manufacture but can be extended by a Member State for the regulation of blood components. An overview is provided in the Reports of the European Committee (Partial Agreement) on Blood Transfusion (CD-P-TS). The report indicates that in addition to the serological standard tests that are obligatory for the testing of plasma for fractionation (summarized in *Table 2*), testing for HIV antigen, anti-HBc antibodies, HCV antigen, anti-HTLV-I, and anti-HTLV-II antibodies is required in some Member States, and testing for antibodies against cytomegalovirus is performed in certain cases. However, these additional rules are applicable only if blood components for transfusion (erythrocytes, platelets, or plasma) are produced.

The test regime required for donations used for production of plasma derivatives are summarized in the *Ph. Eur.* monograph *Human Plasma for Fractionation (0853)*. Only licensed tests or test kits can be used for donor screening. Licensed tests have a CE mark, which confirms that the quality of the test meets predefined criteria (e.g., an HBV screening test must detect HBsAg in a concentration of 0.5 ng/mL or less). The most important quality attributes, namely specificity and sensitivity, are tested in clinical trials using donor samples (minimum of 5000 samples) and clinical samples (minimum of 200 samples). Sensitivity of the tests must be demonstrated with positive samples (minimum of 400 samples) and with seroconversion panels (minimum of 20 panels).

HBsAg tests and antibody tests against HIV Types 1 and 2 also are used for the testing of fractionation pools. The plasma manufacturer should demonstrate that the test is qualified for this use and meets the requirements laid down in the appropriate guidelines of the European Medicines Agency.

The current test regime is discussed regularly among EU Member States and may be subject to change if necessary because of the epidemiological situation. If changes in the requirements are made, the *Ph. Eur.* monograph *Human Plasma for Fractionation (0853)* and the product-specific monographs will be adopted accordingly (e.g., a change in the monograph *Human Plasma (Pooled and Treated for Virus Inactivation) (1646)* is proposed; it requires testing for HEV by NAT).

NAT Tests

As described previously, only licensed serological tests that use antibody- or antigen-detection technology are required to screen plasma in single-donation format. However, NAT testing generally can detect evidence of viral infection at an early stage, and FDA licensed NAT tests for HIV-1 and HCV in 2001 for source plasma donors and in 2002 for whole-blood collections. Thus NAT tests are used to screen plasma donations, generally in a minipool format, using pool sizes that depend on the analytical sensitivity of the NAT test and, in some cases (HCV and B19V), the fractionation pool. In general, plasma donations are screened in a minipool format in which the pool size depends on the analytical sensitivity of the NAT test. The size of the minipool used for source plasma donations generally is much larger than that for blood donation testing (as large as 512 compared with 96 for blood donations). The turnaround time required for retesting a reactive pool in order to identify the reactive donation is less critical for plasma compared with that for blood for transfusion because some blood components such as platelets have a short shelf life.

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FDA REQUIREMENTS OR RECOMMENDATIONS

To adequately and appropriately reduce the risk of transmissions of HIV-1, HCV, and HBV, FDA-licensed NAT tests are required for donor screening. A list of FDA-licensed, donor-screening NAT tests and serological tests is updated as needed and is available on the FDA website.

FDA's initial guidance for HIV NAT in 1999 recommended standards for the manufacture and clinical evaluation of tests to detect nucleic acid sequences of HIV-1 and HIV-2 for licensure. This guidance provided some of the major regulatory and scientific guidance for NAT assays not only for HIV but also for other transfusion-transmitted viruses. Since then FDA has revised the requirements for the analytical sensitivity of HIV-1 and HCV NAT tests as 100 IU/mL for HIV-1 RNA and HCV RNA when tested in a minipool or as 10,000 IU/mL HIV-1 RNA or 5000 IU/mL HCV RNA when tested in an individual donation. FDA's 2004 guidance on NAT screening of HIV-1 and HCV in donor whole blood, blood components, and source plasma and a further guidance in 2010 contain recommendations about testing, product disposition, and donor deferral and reentry. The latter supersedes earlier recommendations for reentry of donor deferral and reentry because of serological testing results for anti-HIV-1 and anti-HCV.

The source plasma industry has voluntarily implemented HBV NAT testing in minipool format. Several FDA-licensed HBV NAT tests for donor screening are available. In 2012 FDA finalized a guidance recommending the use of HBV NAT on pooled and individual samples from donors of whole blood and blood components for transfusion or for further manufacture, including recovered plasma and source plasma. The guidance recommends an NAT test sensitivity of 100 IU/mL for testing individual donations of whole blood and blood components intended for transfusion. Because of the virus-reduction step(s) used during the manufacturing of plasma-derived products and the presence of neutralizing anti-HBsAg in the manufacturing pools, FDA recommends a NAT test sensitivity of 500 IU/mL for individual donations when manufacturers test minipools of plasma for further manufacture. The guidance also contains recommendations about product testing and disposition, donor management, methods of donor requalification, and product labeling. The guidance also supersedes the relevant recommendations based on HBsAg and anti-HBc serological testing results.

In 2009 FDA issued a final guidance for B19V NAT testing following a postmarket surveillance study report of a B19V transmission incident associated with solvent and detergent-treated (S/D-treated) pooled plasma. The guidance recommends the use of B19V NAT as an in-process test for plasma for further manufacturing to ensure that the level of B19V DNA in fractionation pools does not exceed 10^4 IU/mL. The guidance document recommends that the primers and probes selected for a B19V NAT test should detect all known genotypes of the virus. The WHO International B19 Genotype Panel containing three genotypes is available for validation purposes. Currently, in-process HAV NAT testing is widely implemented by fractionators who use source plasma as starting plasma, but FDA has not issued a relevant guidance document. Because the in-process B19V NAT test is used to limit the virus load in the plasma pool, these tests must be capable of B19V DNA quantitation (unlike the NAT tests for HBV, HCV, HIV, and HAV, which are qualitative NAT tests).

EUROPEAN REQUIREMENTS

NAT tests used for donor screening are subject to licensing and receive the CE mark if a test meets the predefined test specifications. NAT tests for plasma pool samples must be validated according to the *Ph. Eur.* general test *Nucleic Acid Amplification Techniques (20621)*. Currently, plasma for manufacture (plasma pools for fractionation) must be tested for HCV RNA by a NAT test, but there are no requirements for NAT testing for HBV DNA and HIV RNA although most plasma manufacturers voluntarily test for all three viruses. The guideline requires that a test should be able to detect all HCV genotypes. However, in view of the difficulty of obtaining rare HCV genotypes, it is sufficient that at least the most prevalent genotypes (in Europe, genotypes 1 and 3) are detected at a suitable level. Plasma should be negative when screened with a test that can detect a sample containing 100 IU/mL of HCV RNA (calibrated against the WHO HCV International Standard).

Testing for B19V DNA generally is not required but must be performed for products that are seen as a higher risk for patients if this virus is present (e.g., anti-D immunoglobulin products and plasma that is pooled and inactivated by S/D treatment). In addition, the latter product also must be tested and found nonreactive for HAV RNA, and in the future it also should be nonreactive for HEV RNA. Unlike testing for HCV and HAV where the fractionation pool should be nonreactive for these viruses, for B19V testing the virus load in the fractionation pool and pooled S/D-treated plasma should not exceed 10 IU/µL B19V DNA. The requirements are detailed in the product-specific *Ph. Eur.* monographs. In order to avoid a reactive pool, which would have to be discarded, NAT testing also is performed on single donations or preferentially on minipools comprising 16–512 donations. Although the requirement for B19V and HAV NAT testing is applicable only to plasma used for the manufacture of pooled S/D-treated plasma and anti-D immunoglobulin products, most plasma manufacturers voluntarily test plasma destined for manufacture of all plasma-derived products to reduce the virus load in the fractionation pools.

The current FDA and EU requirements for testing plasma for further manufacture are summarized in *Table 2* and *Table 3*.

Table 2. FDA and EU Serology Testing Requirements for Plasma for Further Manufacture

Screening Test	FDA	EU
Serological Testing of Individual Plasma Donations (Recovered and Source)		
HBsAg	Required	Required
Anti-HBc	Not required	Not required
Anti-HIV-1/Anti-HIV-2	Required	Required
Anti-HTLV-I/II	Not required	Not required
Anti-HCV	Required	Required
Serological Testing of the Fractionation Pool		

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Table 2. FDA and EU Serology Testing Requirements for Plasma for Further Manufacture *(continued)*

Screening Test	FDA	EU
HBsAg	Not required but widely implemented by plasma fractionators	Required
Anti-HIV	Not required but widely implemented by plasma fractionators	Required

Table 3. FDA and EU NAT Testing Requirements for Plasma for Further Manufacture

Screening Test	FDA	EU
NAT Testing of Plasma Donations in Minipool Format		
HIV-1 RNA	Required, using tests with a sensitivity of 10,000 IU/mL for the individual donation	Not required but widely implemented by plasma fractionators
HCV RNA	Required, using tests with a sensitivity of 5000 IU/mL for the individual donation	Not required but recommended in order to avoid unnecessary loss of a fractionation pool (see below)
WNV RNA	Not required	Not required
HBV DNA	Required, using tests with a sensitivity of 500 IU/mL for the individual donation	Not required but widely implemented by plasma fractionators
B19V DNA	Required with a manufacturing pool limit of $\leq 10^4$ IU/mL B19V DNA	Required for specific products (anti-D immunoglobulin and pooled S/D-treated plasma); a manufacturing pool limit of B19V DNA ≤10 IU/µL is required. This limit is voluntarily implemented by most plasma fractionators for all products.
HAV RNA	Not required but widely implemented by plasma fractionators	Required only for S/D-treated plasma; not required for other products but widely implemented by most plasmafractionators
HEV	Currently not required	Testing not yet required; testing requirements will be introduced for a specific product only (pooled S/D-treatedplasma).
NAT Testing of the Fractionation Pool		
HIV-1 RNA	Not required but widely implemented by plasma fractionators	Not required but widely implemented by plasma fractionators
HCV RNA	Not required but widely implemented by plasma fractionators	Required; the fractionation pool must be nonreactive using a test that detects 100 IU/mL of HCV RNA.
WNV RNA	Not required	Not required
HBV DNA	Not required but widely implemented by fractionators	Not required but widely implemented by fractionators
B19V DNA	Required; a limit of $\leq 10^4$ IU/mL B19V DNA for fractionation pools is required.	Required for specific products (anti-D immunoglobulin and pooled S/D-treated plasma); a limit of B19V DNA ≤10 IU/µL for fractionation pools is required. This limit is voluntarily implemented by most plasma fractionators for all products.
HAV RNA	Not required but widely implemented by plasma fractionators	Required only for a specific product (pooled S/D-treated plasma); the fractionation pool must be nonreactive using a test that detects 100 IU/mL.
HEV	Not required	Testing not yet required; testing requirements will be introduced for a specific product only (pooled S/D-treated plasma). After the requirement is implemented, the plasma pool must be nonreactive using a test that can detect 2.5 $\log_{10}$ IU/mL of HEV RNA[a].

[a] The new monograph will be implemented soon. See the draft *Ph. Eur.* monograph *Human Plasma (Pooled and Treated for Virus Inactivation (1640))*. http://pharmeuropa.edqm.eu/TextsForComment/NetisUtils/srvrutil_getdoc.aspx/2L3OqDZGmCLmnDZGsHIveT6q0//1646E.pdf. Accessed 20 February 2013.

CONCLUSIONS

All the measures discussed in this chapter, along with virus-reduction steps included during the manufacturing process, ensure the safety of plasma-derived products. However, testing of plasma for further manufacture is only one of the steps taken to ensure the safety of the final plasma-derived products. Both manufacturers and regulators face continuing challenges because of the emergence of new blood-borne viruses, mutants, and variants of existing viruses not detected by current serological/NAT technology. The development of new screening tests and regulatory guidance documents depends on whether the emerging virus is a risk to the safety of plasma-derived products.

APPENDIX

Regulatory Guidances

- GAO-HEHS-98-205: General Accounting Office, Blood Plasma Safety: Plasma product risks are low if good manufacturing practices are followed.
- 21 CFR 606: Current good manufacturing practice for blood and blood components.

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⟨1241⟩ WATER–SOLID INTERACTIONS IN PHARMACEUTICAL SYSTEMS

INTRODUCTION

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia.* These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (•,) to specify this fact.

Pharmaceutical solids as raw materials or as constituents of dosage forms most often come in contact with water during processing and storage. This may occur (a) during crystallization, lyophilization, wet granulation, or spray drying; and (b) because of exposure upon handling and storage to an atmosphere containing water vapor or exposure to other materials in a dosage form that contain water capable of distributing it to other ingredients. Some properties known to be altered by the association of solids with water include rates of chemical degradation in the "solid-state", crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compactibility, compact hardness, and microbial contamination.

Although precautions can be taken when water is perceived to be a problem, i.e., eliminating all moisture, reducing contact with the atmosphere, or controlling the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognize that there are many situations where a certain level of water in a solid is required for proper performance, e.g., powder compaction. It is essential for both reasons, therefore, that as much as possible is known about the effects of moisture on solids before strategies are developed for their handling, storage, and use.

Some of the more critical pieces of required information concerning water–solid interactions are:

- total amount of water present;
- the extent to which adsorption and absorption occur;
- whether or not hydrates form;

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- specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperature;
- site of water interaction, the extent of binding, and the degree of molecular mobility;
- effects of temperature and relative humidity;
- essentially irreversible hydration;
- kinetics of moisture uptake;
- various factors that might influence the rate at which water vapor can be taken up by a solid;
- for water-soluble solids capable of being dissolved by the sorbed water, under which conditions dissolution will take place.

PHYSICAL STATES OF SORBED WATER

Water can physically interact with solids in different ways. It can interact at the surface (adsorption) or it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity. Absorption is characterized by an association of water per gram of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that is generally independent of the specific surface area.

Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be non-stoichiometric. Upon dehydration, crystal hydrates may either retain their original crystal structure, lose their crystallinity and become amorphous, or transform into a new anhydrous or less-hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water because there is sufficient molecular disorder in the solid to permit penetration, swelling, or dissolution. Such behavior is observed with most amorphous polymers and with small-molecular-mass solids rendered amorphous during preparation, e.g., by lyophilization, or after milling. The introduction of defects into highly crystalline solids will also produce this behavior. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of two states: "glassy" or "fluid"; the temperature at which one state transforms into the other is the glass transition temperature, T_G.

Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticizer and reduce the value of T_G. Because the rheological properties of "fluid" and "glassy" states are quite different, i.e., the "fluid" state exhibits much less viscosity as the temperature rises above the glass transition point, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Because amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-mass materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a "fluid" state. This is the basis of "cake collapse" often observed during the lyophilization process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e., to dissolve in their own sorbed water, at relative humidities, RH_I, in excess of the relative humidity of a saturated solution of the solid, RH_0. Deliquescence arises because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as RH_I is greater than RH_0.

The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a state that is directly bound to the solid, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heat of sorption, freezing point, nuclear magnetic resonance, dielectric properties, and diffusion.

Such changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought as unavailable to affect the properties of the solid, whereas larger amounts of sorbed water may become more clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water–solid interactions. Recognizing that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of T_G. Hence, water is "frozen" into the solid structure and is rendered immobile by the high viscosity, e.g., 10^{13} Pa · s. As the amount of water sorbed increases and T_G decreases, approaching ambient temperatures, the glassy state approaches that of a "fluid" state and water mobility along with the mobility of the solid itself increases significantly. At high RH, the degree of water plasticization of the solid can be sufficiently high so that water and the solid can now achieve significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticizer.

Rates of Water Uptake

The rate and extent to which solids exposed to the atmosphere might either sorb or desorb water vapor can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure,

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therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled with some contributions from heat-transfer mechanisms. Thus, factors such as vapor diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role. Indeed, the method used to make measurements can often be the rate-determining factor because of these environmental and geometric factors.

DETERMINATION OF SORPTION–DESORPTION ISOTHERMS

Principle

The tendency to take up water vapor is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e., equilibrium. Relative humidity, RH, is defined by the following equation:

$$RH = (P_C \times 100)/P_0$$

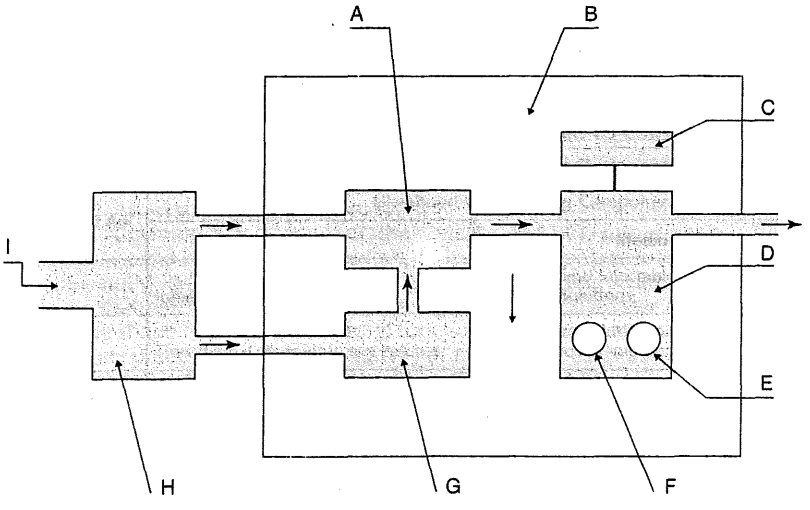
P_C = pressure of water vapor in the system

P_0 = saturation pressure of water vapor under the same conditions

The ratio P_C/P_0 is referred to as the relative pressure. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. As the name indicates, the sorption–desorption isotherm is valid only for the reference temperature, hence a special isotherm exists for each temperature. Ordinarily, at equilibrium, moisture content at a particular relative humidity must be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption–desorption hysteresis.

Methods

Samples may be stored in chambers at various relative humidities. The mass gained or lost for each sample is then measured. The major advantage of this method is convenience, while the major disadvantages are the slow rate of reaching constant mass, particularly at high relative humidities, and the error introduced in opening and closing the chamber for weighing. Dynamic gravimetric water sorption systems allow the on-line weighing of a sample in a controlled system to assess the interaction of the material with moisture at various programmable levels of relative humidity at a constant temperature. The major benefit of a controlled system is that isothermal conditions can be more reliably established and that the dynamic response of the sample to changing conditions can be monitored (see *Figure 1*).



A. Humidity controller
B. Temperature controlled chamber
C. Balance module
D. Humidity regulated module
E. Reference
F. Sample
G. Vapour humidifier
H. Flow control module
I. Dry gas

Figure 1. Example of an apparatus for the determination of the water sorption (other designs are possible).

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Data points for the determination of the sorption isotherm (e.g., from 0% to approximately 95% RH, noncondensing) are only taken after a sufficiently constant signal indicates that the sample has reached equilibrium at a given level of humidity. In some cases (e.g., deliquescence), the maximum time may be restricted although the equilibrium level is not reached. The apparatus must adequately control the temperature to ensure a good baseline stability as well as accurate control of the relative humidity generation. The required relative humidities can be generated, e.g., by accurately mixing dry and saturated vapor gas with flow controllers. The electrostatic behavior of the powder must also be considered. The verification of the temperature and the relative humidity (controlled with, for example, a certified hygrometer, certified salt solutions, or deliquescence points of certified salts over an adequate range) must be consistent with the instrument specification. The balance must provide a sufficient mass resolution and long-term stability.

It is also possible to measure amounts of water uptake not detectable gravimetrically using volumetric techniques. In the case of adsorption, to improve sensitivity, the specific surface area of the sample can be increased by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid does not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity. For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to get desolvation of the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, care must be taken to note any adverse effects this might have on the solid such as dehydration, chemical degradation, or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out because of these possible pitfalls.

Report and Interpretation of the Data

Sorption data are usually reported as a graph of the apparent mass change in percent of the mass of the dry sample as a function of relative humidity or time. Sorption isotherms are reported both in tabular form and as a graph. The measurement method must be traceable with the data.

Adsorption–desorption hysteresis can be interpreted, for example, in terms of the porosity of the sample, its state of agglomeration (capillary condensation), the formation of hydrates, polymorphic change, or liquefying of the sample. Certain types of systems, particularly those with microporous solids and amorphous solids, are capable of sorbing large amounts of water vapor. Here, the amount of water associated with the solid as relative humidity is decreased, is greater than the amount that originally sorbed as the relative humidity was increased. For microporous solids, vapor adsorption–desorption hysteresis is an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they "fill" (adsorption) and "empty" (desorption) under different equilibrium conditions. For nonporous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapor–solid interaction due to a change in the equilibrium state of the solid, e.g., conformation of polymer chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption. In measuring sorption–desorption isotherms, it is therefore important to establish that something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, because one is usually dealing with a polymer plasticized into its "fluid" state, where the solid is undergoing significant change.

In the case of crystal hydrate formation, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure and the amount of water taken up will usually exhibit a stoichiometric mole:mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems.

For situations where water vapor adsorption occurs predominantly, it is very helpful to measure the specific surface area of the solid by an independent method and to express adsorption as mass of water sorbed per unit area of solid surface. This can be very useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5% m/m uptake of water could hardly cover the bare surface of 100 m^2/g, while for 1.0 m^2/g this amounts to 100 times more surface coverage. In the case of pharmaceutical solids that have a specific surface area in the range of 0.01 m^2/g to 10 m^2/g, what appears to be low water content could represent a significant amount of water for the available surface. Because the "dry surface area" is not a factor in absorption, sorption of water with amorphous or partially amorphous solids can be expressed on the basis of unit mass corrected for crystallinity, when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

DETERMINATION OF THE WATER ACTIVITY

Principle

Water activity, A_W, is the ratio of vapor pressure of water in the product (P) to saturation pressure of water vapor (P_0) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point, or from indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed. Ignoring activity coefficients, the relationship between A_W and equilibrium relative humidity (ERH) are represented by the following equations:

$$A_W = P/P_0$$

$$ERH(\%) = A_W \times 100$$

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Method

The water activity is determined by placing the sample in a small airtight cup inside which the equilibrium between the water in the solid and the headspace can be established. The volume of the headspace must be small in relation to the sample volume in order not to change the sorption state of the sample during the test. The equilibration as a thermodynamic process takes time but may be accelerated by forced circulation within the cell. The acquired water activity value is only valid for the simultaneously determined temperature. This requires a precise temperature-measuring device as part of the equipment. Furthermore, the probe must be thermally insulated to guarantee a constant temperature during the test. The sensor measuring the humidity of the headspace air above the sample is a key component. Theoretically, all types of hygrometers can be used, but for analytical purposes miniaturization and robustness are a precondition. The A_W measurement may be conducted using the dew point/chilled mirror method.[1] A polished, chilled mirror is used as a condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations.

These instruments are typically calibrated over an adequate range, for example, using some saturated salt solutions at 25° such as those listed in *Table 1*.

Table 1. Standard Saturated Salt Solutions

Saturated Salt Solutions at 25°	ERH (%)	A_W
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753
Magnesium nitrate ($Mg(NO_3)_2$)	52.9	0.529
Magnesium chloride ($MgCl_2$)	32.8	0.328
Lithium chloride ($LiCl_2$)	11.2	0.112

⟨1251⟩ WEIGHING ON AN ANALYTICAL BALANCE

INTRODUCTION

Weighing is a frequent step in analytical procedures, and the balance is an essential piece of laboratory equipment. The general information described here applies directly to electronic balances used in analytical procedures. Although many portions of the chapter are applicable to all balances, some are applicable only to analytical balances. This chapter should not be considered all-inclusive, and other sources of information (e.g., the US National Institute of Science and Technology and balance manufacturers) may be useful and applicable when analysts perform a weighing operation or implement a weighing procedure. The information given in this chapter is applicable not only to balances used for materials that must be accurately weighed (see *Balances* ⟨41⟩) but also to balances used in all analytical procedures.

QUALIFICATION

Users should consult *Analytical Instrument Qualification* ⟨1058⟩, standard operating procedures, and recommendations from manufacturers when they devise qualification plans.

Installation

The balance's performance depends on the conditions of the facility where it is installed. Analysts should consult information provided by the manufacturer before they install a balance.

SUPPORT SURFACE

The balance should be installed on a solid, level, nonmagnetic surface that minimizes the transmission of vibration (e.g., a floor-mounted, granite weigh bench). If a metallic support surface is used, the surface should be grounded in order to prevent the buildup of static electricity.

[1] AOAC International Official Method 978.18.

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LOCATION

If possible, the balance should be located in a room that is temperature and humidity controlled. The location should have a clean, consistent electrical power supply. The location should be free of drafts and should not be near ovens, furnaces, air conditioner ducts, or cooling fans from equipment or computers. The balance should be positioned away from outside windows so that direct sunlight does not strike the balance. The balance should not be installed near sources of electromagnetic radiation such as radio-frequency generators, electric motors, or hand-held communication devices (including cordless telephones, cellular telephones, and walkie-talkies). The balance should not be located near magnetic fields induced by laboratory instrumentation or other equipment.

The performance of the balance should be assessed following installation and before use in order to demonstrate adequate performance. In some situations, it may not be possible to position the balance in an optimum environment. Examples of potential facility issues include the following:

1. Air currents sometimes are present in the laboratory.
2. Temperatures in the laboratory vary excessively (check the manufacturer's literature about temperature sensitivity).
3. Humidity is either very low or very high. Either condition may increase the rate at which the sample weight varies because of pickup or loss of water. Low humidity increases the buildup of static electricity.
4. Adjacent operations are causing vibration.
5. Corrosive materials are used nearby or are routinely weighed.
6. The balance is located within a fume hood because it is used to weigh corrosive or hazardous materials.
7. The balance is adjacent to equipment that produces a magnetic field (e.g., a magnetic stirrer).
8. Direct sunlight strikes the balance.

In situations when the balance is located near equipment or systems that induce vibration, drafts, electromagnetic radiation, magnetic fields, or changes in temperature or humidity, the assessment should be conducted with those systems operating in order to duplicate a worst-case scenario.

Operational Qualification

An operational qualification should be performed either by the user or by a qualified third-party vendor after the equipment has been installed.

As a minimum, the power should be turned on and the balance should be allowed to equilibrate according to the manufacturer's instructions (1–24 h, depending on the type of balance) before use. Depending on the balance, analysts should include the following procedures in the operational qualification:

1. Mechanical mobility of all moveable parts
2. Control of stable indication
3. Manually triggered or automatic adjustment by means of built-in weights
4. Operation of ancillary equipment
5. Tare function
6. Initial calibration

Several types of electronic analytical balances use built-in weights for manually triggered or automatic adjustment. This adjustment usually is applied to reduce the drift of the balance over time and to compensate for drifts caused by variations in the ambient temperature.

Calibration normally is performed as part of the operational qualification, but it also can be performed periodically thereafter. Calibration should be performed at the location where the balance is used in normal operation.

Performance Qualification

Table 1 provides a list of the most important balance properties that should be assessed during performance qualification. Depending on the risk of the application and the required weighing process tolerance, some of these tests may be omitted. Tests also can be omitted if there is evidence that the property in question has only minimal effect on the weighing performance. Any procedures used should be consistent with in-house standard operating procedures, applicable for the specific balance, and adequately justified. Performance qualification should be performed periodically as described in standard operating procedures, and the frequency of each of the individual tests can vary depending on the criticality of the property.

The weights that are used to perform the tests should be stored and handled in a manner that minimizes contamination. Before executing the tests, the technician should place the weights in the vicinity of the balance for an appropriate time to reach sufficient thermal equilibrium. If possible, tests should be carried out with a single test weight in order to minimize handling errors, but multiple test weights are permitted.

The tests should be recorded in such a manner that the data can be used to easily track balance performance and to assist in laboratory investigations as needed. Meaningful acceptance criteria can be set depending on the required weighing tolerance, i.e., the maximum allowed deviation permitted by specifications, regulations, etc., of a quantity to be weighed from its target value. Procedures should be in place to address test results that are outside acceptable ranges and to provide assurance that balance cleanliness and environment have not affected the result. Also, a procedure should be in place for removing a balance from operation when observed results fall outside acceptable ranges.

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Table 1. Suggested Performance Tests and Acceptance Criteria

Property	Definition	Examples	Acceptance Criteria
Sensitivity	Change in the displayed value divided by the load on the balance, which causes this change.	The test load at or sufficiently close to the capacity of the balance.	NMT 0.05% deviation from 1 (the sensitivity of a correctly adjusted balance), where ⟨41⟩ is applicable. For other uses, respective tolerance requirement divided by 2.
Linearity	Ability of a balance to follow the linear relationship between a load and the indicated weighing value. Nonlinearity usually is expressed as the largest magnitude of any linearity deviation within the test interval.	From 3 to 6 points over the range of the balance.	NMT 0.05% deviation where ⟨41⟩ is applicable. For other uses, respective tolerance requirement divided by 2.
Eccentricity	Deviation in the measurement value caused by eccentric loading—in other words, the asymmetrical placement of the center of gravity of the load relative to the load receiver. Eccentricity usually is expressed as the largest magnitude of any of the deviations between an off-center reading and the center reading for a given test load.	Performed in the center of gravity and the four quadrants (for rectangular platter shapes) or at analogous locations for other platter shapes. Test load usually should be 30% of the capacity of the balance or higher (refer to the manufacturer's manual for any possible upper limit).	NMT 0.05% deviation where ⟨41⟩ is applicable. For other uses, respective tolerance requirement divided by 2.
Repeatability	Ability of a weighing instrument to display identical measurement values for repeated weighings of the same objects under the same conditions, e.g., the same measurement procedure, same operator, same measuring system, same operating conditions, and same location over a short period of time. Repeatability usually is expressed as the standard deviation of multiple weighings.	10 replicate weighings (using a test weight that is a few percent of the nominal capacity of the balance).	Requirement from ⟨41⟩ where applicable. For other uses, user specified requirements will apply.

Sensitivity, linearity, and eccentricity all account for systematic deviations; i.e., they limit the accuracy of the balance (based on the definition of accuracy in *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2). In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization, this concept is referred to as trueness. Because deviations are largely independent from each other, it is not likely that all deviations occur simultaneously and have the same algebraic sign. Therefore the arithmetic addition of all individual deviations to assess the balance accuracy would constitute a rather conservative approach. A quadratic addition of the individual deviations is a more realistic approach. By allocating 50% of the weighing tolerance budget to the acceptance criteria of the individual properties, e.g., sensitivity, linearity, and eccentricity, analysts ensure adherence to the required weighing tolerance. Therefore, the acceptance criteria for the individual properties that account for the systematic deviations are set to weighing tolerance divided by 2. These properties—or a subset of them—also can be taken to fulfill the accuracy requirement described in ⟨41⟩. In this case the acceptance criteria thus allow a maximum deviation of 0.05% for sensitivity, linearity, and eccentricity. Repeatability preferably is tested with a test weight of a few percent of the balance capacity. At the lower end of its measurement range, the performance of laboratory balances is limited by the finite repeatability, and limitations induced by systematic deviations normally can be neglected. Therefore, the whole weighing tolerance budget can be allocated to the acceptance criterion of the repeatability test.

For the sensitivity and linearity tests as described above, the analyst should use certified weights with an appropriate weight class (e.g., according to International Organization of Legal Metrology R111 or American Society for Testing and Materials E617, available from www.oiml.org and www.astm.org, respectively). [NOTE—If a differential method is used for the linearity test, certified weights may not be required.]

Depending on the acceptance criterion, it may be sufficient to consider only the nominal weight value of the test weights. If the nominal value of the test weight is considered, analysts should ensure that the maximum permissible error does not exceed one-third of the acceptance criterion. Alternatively, if the certified value of the test weight is considered, its calibration uncertainty should not exceed one-third of the acceptance criterion. If more than one weight is used to perform the test, the calibration uncertainties of the weights must be summed and the sum should not exceed one-third of the acceptance criterion. For tests such as eccentricity or repeatability, the use of certified weights is optional, but analysts must ensure that the mass of the weight does not change during the test.

The tests described above also can be included in formal periodic calibration in order to fulfill applicable cGMP requirements.

Balance Checks

A balance check using an external weight helps ensure that the balance meets weighing tolerance requirements. The balance check is performed at appropriate intervals based on applicable standard operating procedures. The frequency of the balance check depends on the risk of the application and the required weighing tolerance. Checks with external weights can be replaced partially using automatic or manually triggered adjustment by means of built-in weights. When analysts perform the balance check with an external weight, the same acceptance criteria may apply as described in the sensitivity test above.

Minimum Weight

The minimum net sample weight, in short, minimum weight, m_{min}, of an analytical balance can be expressed by the equation:

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$$m_{min} = k \times s/\text{required weighing tolerance}$$

where k is the coverage factor (usually 2 or larger) and s is the standard deviation (in a mass unit, e.g., in mg) of NLT 10 replicate measurements of a test weight. If the standard deviation obtained is less than $0.41d$, where d is the scale interval, the standard deviation is replaced by $0.41d$. The lower limit of $0.41d$ for the standard deviation results from the rounding error of the digital indication of a weighing instrument. The rounding error that is allocated to a single reading is calculated as $0.29d$. Note that a weighing always consists of two readings, one before and one after placing/removing the sample on/from the pan, with the difference between the two indications being the net sample weight. The two individual rounding errors are usually added quadratically, leading to $0.41d$. Taring the instrument after placing the tare container on the pan does not affect the rounding error as the zero indication is also rounded. The minimum weight describes the lower limit of the balance below which the required weighing tolerance is not adhered to. The equation above takes into account that the performance of analytical balances at the lower end of the measurement range is limited by the finite repeatability.

For materials that must be accurately weighed, ⟨41⟩ stipulates that repeatability is satisfactory if two times the standard deviation of the weighed value, divided by the desired smallest net weight (smallest net weight that the users plan to use on that balance), does not exceed 0.10%. For this criterion the equation above simplifies to:

$$m_{min} = 2000 \times s$$

If the standard deviation obtained is less than $0.41d$, where d is the scale interval, the standard deviation is replaced by $0.41d$.

If not subject to the requirements of ⟨41⟩, the minimum weight value may vary depending on the required weighing tolerance and the specific use of the balance.

To facilitate handling, the test weight that is used for the repeatability test does not need to be at the minimum weight value but can be larger because the standard deviation of repeatability is only a weak function of the test weight value.

In order to satisfy the required weighing tolerance, when samples are weighed the amount of sample mass (i.e., the net weight) must be equal to or larger than the minimum weight. The minimum weight applies to the sample weight, not to the tare or gross weight.

Factors that can influence repeatability while the balance is in use include:

1. The performance of the balance and thus the minimum weight can vary over time because of changing environmental conditions.
2. Different operators may weigh differently on the balance—i.e., the minimum weight determined by different operators may be different.
3. The standard deviation of a finite number of replicate weighings is only an estimation of the true standard deviation, which is unknown.
4. The determination of the minimum weight with a test weight may not be completely representative for the weighing application.
5. The tare vessel also may influence minimum weight because of the interaction of the environment with the surface of the tare vessel.

For these reasons, when possible, weighings should be made at larger values than the minimum weight, i.e., the desired smallest net weight that the users plan to use on that balance should be larger than the minimum weight.

OPERATION OF THE ANALYTICAL BALANCE

Select the appropriate balance for the quantity and performance needed. General chapter ⟨41⟩ provides requirements for balances used for materials that must be accurately weighed. The balance user should check the balance environment (vibration, air currents, and cleanliness) and status of calibration before use.

Receivers

To ensure suitable performance in measuring the weight of a specimen, analysts should consider selection of a proper receiver for the material.

GENERAL CHARACTERISTICS

All receivers must be clean, dry, and inert. The total weight of the receiver plus the specimen must not exceed the maximum capacity of the balance. With a properly maintained and adjusted laboratory balance, weighing uncertainty for small samples, i.e., net weights with a mass not exceeding typically a few percent of the capacity of the balance, essentially is determined by the repeatability. However, repeatability depends on the size and surface area of the weighed object. For this reason large or heavy receivers introduce a deviation from the conditions under which the repeatability was determined without considering the receivers. Therefore, either receivers of a low mass and small surface should be used (especially in cases when specimens of low weight are being measured) or the repeatability test should be performed with the receiver placed on the weighing pan as a preload. Receivers should be constructed from nonmagnetic materials in order to prevent magnetic interference with electronic balance components. Receivers should be used at ambient temperature in order to prevent the formation of air currents within the weighing chamber.

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SOLID SAMPLES

Receivers for weighing solid materials include weighing paper, weighing dishes, weighing funnels, or enclosed vessels, including bottles, vials, and flasks. Hygroscopic papers are not recommended for weighing because they may have a detrimental effect on the observed results.

Weighing dishes typically are constructed from a polymer or from aluminum. Antistatic weighing dishes are available for measuring materials that retain static electricity. Weighing funnels typically are constructed from glass or from a polymer. The design of this type of receiver combines attributes of a weighing dish and a transfer funnel, which can simplify the analytical transfer of a weighed powder to a narrow-necked vessel such as a volumetric flask. For solid samples that are volatile or deliquescent, analysts must weigh the material into an enclosed vessel. Where practical, analysts should use an enclosed vessel with a small opening in order to reduce sample weight loss from volatilization or weight gain from the adsorption and absorption of atmospheric water.

LIQUID SAMPLES

Receivers for liquid samples typically are inert, enclosed vessels. For liquid samples that are volatile or deliquescent, analysts should use an enclosed vessel with a small opening, and the enclosure should be replaced rapidly following material transfer. Special precautions should be taken to be certain that the receiver and the enclosure are constructed from a material that is compatible with the liquid sample. The receiver and enclosure must have a seal that is sufficient to prevent leaks from a liquid that is of low viscosity or has low surface tension or a low boiling point.

Types of Weighing

WEIGHING FOR QUANTITATIVE ANALYSIS

The initial step for many quantitative analyses is to accurately weigh a specified amount of a sample. *General Notices, 6.50.20 Solutions* stipulates that solutions for quantitative measures must be prepared using accurately weighed analytes: i.e., analysts must use a balance that meets the criteria in ⟨41⟩. Errors introduced during the weighing of a sample can affect the accuracy of all subsequent analytical measurements.

ADDITION WEIGHING

Addition weighings typically are used for solid samples or liquid samples for which volatility is not an issue. The receiver is placed on the balance. After the balance display stabilizes, the analyst should tare the balance; add the desired amount of material to the receiver; allow the balance display to stabilize; record the weight; and quantitatively transfer the material to an appropriate vessel or, if it cannot be guaranteed that the entire amount has been transferred, weigh the receiver again and note the weight difference.

DISPENSE WEIGHING

Dispense weighing typically is used for weighing emulsions or viscous liquids such as ointments. In these situations it is not practical to weigh the material into a typical receiver. Accordingly, the analyst should tare the balance; place the sample on the balance in a suitable container (e.g., a bottle, tube, transfer pipet, or syringe) that has been wiped clean on the outside; record the weight after the balance display stabilizes; transfer the desired amount of sample to an appropriate receiving vessel, such as a volumetric flask; and place the pipet or syringe back onto the balance. The difference in the two weighings is equal to the weight of the transferred specimen.

GRAVIMETRIC DOSING

Gravimetric dosing typically is used for sample and standard preparations or capsule filling. For such weighing the analyst places the volumetric flask, vial, or capsule shell on the balance; tares the balance after the balance display stabilizes; adds the solid or liquid components into the receiver by means of dosing units; and records the respective weights.

Problem Samples

ELECTRICALLY CHARGED SAMPLES AND RECEIVERS

Dry, finely divided powders may be charged with static electricity that can make the powder either attracted to or repelled by the receiver or the balance, causing inaccurate weight measurements and specimen loss during transfer. A drift in the balance readings should alert the operator to the possibility that the material has a static charge. Commercially available balances with a built-in antistatic device can be used to remedy the problem. Such devices may use piezoelectric components or a very small amount of a radioactive element (typically polonium) to generate a stream of ions that dissipate the static charge when passed over the powder being weighed. Antistatic weigh boats, antistatic guns, and antistatic screens also are commercially available. The static charge depends also on the relative humidity of the laboratory, which in turn depends on atmospheric conditions. Under certain conditions, static charge is caused by the type of clothing worn by the operator and this charge can cause large errors in the weighing. Borosilicate glassware and plastic receivers have a well-known propensity for picking up static charge, especially at low relative humidity. The gloves used to protect the operator also may increase the potential for a static charge

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problem. Placing the container in a metal holder may help to shield the static charge, and antistatic gloves also can help to alleviate the problem.

VOLATILE SAMPLES

When weighing a liquid that has a low boiling point, analysts must receive the specimen in a vessel with a gas-tight enclosure of small diameter. The analyst then tares the vessel and enclosure, adds the desired amount of sample, and replaces the enclosure. After the balance display stabilizes, the analyst records the specimen weight.

WARM OR COOL SAMPLES

Samples that are warm or cool should be equilibrated in the laboratory, or the weight readings may be erroneous. With regard to warm samples, the apparent weight is smaller than the true weight because of heat convection. For example, a flask that is warmer than ambient air warms up this air, which then flows upward along the flask and reduces the apparent weight of the contents by viscous friction.

HYGROSCOPIC SAMPLES

Hygroscopic materials readily absorb moisture from the atmosphere and steadily gain weight if left exposed. Therefore, hygroscopic samples must be either weighed promptly or placed in a vessel with a gas-tight enclosure. For a gas-tight vessel, analysts should tare the vessel and enclosure, add the desired amount of sample, and replace the enclosure. After the balance display stabilizes, the analyst can record the specimen weight.

ASEPTIC OR BIOHAZARDOUS SAMPLES

The weighing of sterile or biohazardous samples should take place within the confines of a clean bench, biosafety cabinet, isolator, or similar containment device. Air flow within the hood potentially can cause balance instability, so after a balance has been installed under a hood, analysts should perform a rigorous qualification study with suitable weight artifacts (see ⟨41⟩) in order to determine the acceptability of the balance performance in this environment.

WEIGHING CORROSIVE MATERIALS

Many chemicals, such as salts, are corrosive, and materials of this nature should not be spilled on the balance pan or inside the balance housing. Extra care is essential when materials of this nature are weighed. Analysts should consider the use of sealed containers such as weighing bottles or syringes. In the event of a spill, requalification of balance may be necessary, depending on the nature of the spill.

Safety Considerations When Weighing

During a weighing, the analyst may be exposed to high concentrations of a pure substance. The analyst must carefully consider this possibility at all times and should be familiar with the precautions described in the substance's Material Safety Data Sheet before weighing it. Hazardous materials should be handled in an enclosure that has appropriate air filtration. Many toxic—and possibly allergenic—substances present as liquids or finely divided particles. When weighing these substances, analysts should use a mask that covers the nose and mouth to prevent any inhalation of the substance, and they should use gloves to prevent any contact with the skin. [NOTE—The use of gloves is good practice for handling any chemical. If it is necessary to handle the container being weighed, the analyst should wear gloves not only for self-protection but also to prevent moisture and oils from being deposited on the weighed container.]

⟨1265⟩ WRITTEN PRESCRIPTION DRUG INFORMATION—GUIDELINES

The purpose of these guidelines—comprising format, content, and accessibility of prescription drug leaflets—is to help ensure that leaflets are useful. In this context, "useful" means that recipients receive, understand, and are motivated to apply written information about their medicines to achieve maximum benefit and minimize harm. Dispensers, prescribers, health care providers who counsel patients about their medicines, and the patients themselves are intended to be the primary beneficiaries for these guidelines.

CRITERIA (FROM THE KEYSTONE ACTION PLAN[1])

Written prescription medicine information should be based on the following criteria:

1. Scientifically accurate,
2. Unbiased in content and tone,

[1] In December 1996, the "Action Plan for the Provision of Useful Prescription Medicine Information" was presented to the Secretary of Health and Human Services. The plan, commonly known as the "Keystone Plan," described certain criteria for written prescription medicine information. These criteria are described in detail in the action plan, which can be found at www.fda.gov/cder/offices/ods/keystone.pdf.

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3. Sufficiently specific and comprehensive,
4. Presented in an understandable and legible format that is readily comprehensible to consumers,
5. Timely and up-to-date, and
6. Useful.

FORMAT GUIDELINES

1. Group all information from the same category, using brief, clear titles and bullets or subheadings as needed. Avoid symbols and subheadings not directly connected to the information they mark.
2. Be consistent in the placement and labeling of categories of information in all leaflets.
3. Provide information at the sixth-grade reading level or below, if possible (never above eighth-grade level). Do not exclude information to achieve a lower reading level.
4. Use simple, common, accurate terms (for example, use "noise in the ears", not "tinnitus").
5. Use direct language that avoids words with opposite meanings (for example, use "decrease blood pressure", not "increase low blood pressure effect").
6. Provide reasons for instructions (for example, "take with food to avoid upset stomach").
7. Emphasize the most important information. Clearly distinguish warnings from instructions or from other text that may be misinterpreted as warnings.
8. Accompany each pictogram, if used, with corresponding text placed close to the pictogram. Use the simplest pictograms possible. For pictograms intended to prompt patients to ask questions or inform health care providers, add text such as "Tell Doctor" or "Ask Pharmacist".
9. Make text readable by using 12-point or larger type, both uppercase and lowercase letters, an easy-to-read font (for example, a serif font), and adequate space between lines and paragraphs. To call attention to important information, use a larger, boldface type.
10. Evaluate format by performing tests of readability, comprehension, memory, problem solving, and behavioral efficacy and intention, using representative samples of the target population.

CONTENT GUIDELINES

1. Provide enough detail to facilitate correct use, achieve maximum benefit, and minimize harm, including a statement that identifies activities (such as driving or sunbathing) that the patient should avoid.
2. Write text that is unbiased in content and tone and scientifically accurate. The uses described should be consistent with FDA-approved labeling or otherwise permitted by FDA, or should appear in federally recognized drug compendia. Distinguish unlabeled from labeled use.
3. For drugs sold under a brand name, provide both brand and generic names, and include a pronunciation guide for each.
4. Describe the drug and its dosage form. Include indications and contraindications, specific directions for use, what to do if a dose is missed, and what to do in the event of an overdose or poisoning.
5. Do not use abbreviations.
6. Indicate the intended type of benefit (for example, "cure", "prevention", "to help relieve symptoms"). Indicate how—and how soon—the patient should recognize the benefit and what to do if none is observed.
7. Give a balanced evaluation of risks and benefits.
8. List side effects, in order of severity, such as "serious", "most common", and other similar type groupings. It may not be appropriate to provide sufficient detail for the patient to be able to monitor serious or common side effects. Provide guidance to consult the doctor or pharmacist, and indicate that not all the side effects are listed.
9. List sufficiently specific and comprehensive information that includes the provision of all important risk information. Patients should be advised to be sure to inform the provider about all the medicines they are taking.
10. Indicate the potential for therapeutic duplication if the drug is available under multiple names or over-the-counter, or if the active ingredient is contained in other products.
11. If known, include a statement concerning the safety of use in the presence of other conditions and during pregnancy or breast-feeding. Direct affected patients to discuss their condition with health care providers. If the safety of use during pregnancy or breast-feeding has not been established, say so.
12. State whether safety and efficacy have been established in pediatric, geriatric, and other special populations. Patients should be encouraged to discuss with their health care provider any recommendations for dosage adjustment.
13. Illustrate information with diagrams when appropriate. Label the diagram components (for example, device parts) if they are not obvious. The words on the label should be prominently placed thereon with such conspicuousness and in such terms as to render them likely to be read and understood by the ordinary individual under customary conditions of purchase and use.
14. Include the following:
    A. A statement that the product is to be used only by the person for whom it was prescribed,
    B. Storage information,
    C. A completeness disclaimer advising the patient to discuss this issue with the health care provider,
    D. The publisher of the leaflet and the date the leaflet was developed or revised,
    E. Sources of in-depth information and answers to questions, and

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F. Other relevant general statements.

15. The patient should be advised about risks of developing dependence on, or tolerance to, the medication.

ACCESSIBILITY GUIDELINES

1. Write text that is relevant to the intended use of the drug.
2. Design the leaflets to be easy to recognize, consistent in format, and easy to store and retrieve.
3. Supplement the leaflets with oral counseling of patients, including children, the elderly, and caregivers.
4. Include a statement asking the patient to reread the leaflet.
5. Distribute the leaflets with all prescription medicines to consumers (namely, persons independently responsible for any aspect of medicine use or for giving medicines to others).
6. Produce leaflets in Spanish, English, or other languages; and establish criteria for producing them in other languages and for special populations (for example, children, visually handicapped) [NOTE—Ideally, prescription drug information leaflets would be customized for the patient's condition and for other relevant information (for example, gender, age, or physical limitations), and would be available in the patient's primary language. Currently, such customization is neither feasible nor practical, but it remains a goal.]

⟨1285⟩ PREPARATION OF BIOLOGICAL SPECIMENS FOR HISTOLOGIC AND IMMUNOHISTOCHEMICAL ANALYSIS

INTRODUCTION

Histology and immunohistochemistry (IHC) are commonly used to visualize the cellular and biochemical constituents of tissues. Whether the attributes to be probed are detected based on chemical reactivities (histochemistry) or are detected using antibodies (IHC) or lectins with known specificities, the preparation of the specimen for staining is a critical phase of the analysis. Preparation typically involves (1) tissue fixation, (2) mounting the tissue in an embedding medium to permit sectioning that is sufficiently thin for the tissue to be viewed microscopically, and then (3) removal of any embedding medium before histochemical or IHC evaluation.

The purpose of this chapter is to identify factors that should be controlled to optimize the consistency of tissue staining results. The following sections address tissue fixation used to prevent degradation and to prepare the tissue for sectioning and staining. Since these methods are often used to characterize tissue-based therapeutic products and the adequacy of their process methods, USP sometimes includes visual tools to support those product monographs. USP Authentic Visual References (AVRs) are often histology images that have been prepared as described in this chapter and are used as reference standards associated with product monographs. The purpose of histologic analysis is to generate visual images that can be used either for illustration or as a visual reference. When used as AVRs, the image set should include representative images of both failed and passing samples at different magnifications. AVRs can be used to better clarify specifications and acceptance criteria related to, for example, cell content, collagen structure, or integrity.

BASIC PRINCIPLES

The sample tissue must be properly and adequately treated or fixed to limit changes to the extracellular matrix elements or to specific constituents such as cells or proteins. Tissue fixation typically employs a chemical that can rapidly permeate the tissue to effectively cross-link proteins and limit degradation caused by either chemical or enzymatic action. Standard fixation methods typically use 10% neutral buffered histological-grade formalin and are adequate for most, if not all, histochemical analyses. Formalin fixation provides material suitable for IHC analysis provided that proper attention is paid to postfixation treatments (collectively known as antigen retrieval). Once tissue is properly fixed, it can be embedded.

The tissue is embedded in a medium that is sufficiently stiff to maintain sample geometry and the desired orientation for sectioning but is soft enough to be easily and rapidly cut through without distortion. The most common medium used is paraffin, although it is not suitable for nondecalcified hard tissues and other stiff materials (e.g., hard polymers or metals). Because paraffin is not water soluble, tissue generally is passed through a graded series of increasing concentrations of ethanol and finally into xylene (or a suitable substitute) in which the paraffin wax is soluble. Sufficient time is allowed for the paraffin to permeate the tissue, and then the block is sectioned to the desired thickness. Hard tissues commonly are embedded in hard plastics such as poly(methyl methacrylate).

After sectioning, the samples are floated on warm water with or without gelatin so they can be transferred to microscope slides. If the sections are to be used for IHC, it is recommended that gelatin not be used in the water because it can contribute significantly to background staining. Positively charged slides also are recommended to aid in sample retention throughout the IHC processing regime. The sections are allowed to dry in air, in a slide warmer, or in an oven at no more than 50°. Because most histochemical and IHC procedures are carried out under aqueous conditions, the paraffin within the mounted tissue sections must be removed. This is accomplished by reversing the xylene and alcohol rinses until the samples are rinsed in water immediately before staining.

The mounted sections are now ready for histochemical staining. If an immunological staining method is to be used, in most cases an antigen retrieval step will be necessary to break the protein cross-links formed by formalin fixation. Many procedures

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are available to accomplish this, and although heat treatment in acid vapor appears to be effective in recovering the reactivity of most targets in tissues, the ultimate choice may depend on the characteristics of the target to be evaluated.

PROCEDURES—POINTS TO CONSIDER

Fixation

The most common fixatives are aldehydes, alcohols, and oxidizing agents. Each has advantages and disadvantages depending on the particular purpose (see *Table 1*).

Table 1. Attributes of Common Tissue Fixatives

Fixative Name	Mechanism	Advantages	Disadvantages	Notes
Formaldehyde (e.g., 10% neutral buffered formalin)	Cross-links protein	Minimal alteration of protein structure and antigenicity; good penetration	Buffered version is preferred to counteract oxidation of formaldehyde to formic acid, which tends to cause a brown artifact.	Unbuffered precipitates can form, and will be acidic (pH 3–4.6).
Glutaraldehyde (e.g., 0.25%–4%)	Cross-links protein	Morphology suitable for electron microscopy	Deforms α-helix structure of protein, so not good for IHC; slow penetration	Use with 2–3 µm thick sections. Opens structures such as blood vessels to improve access to all tissue surfaces and to prevent opposing surfaces from bonding to each other
Alcohols (e.g., methanol or ethanol)	Denature proteins	Best applied to cytologic smears; good penetration	Can cause tissue hardness and brittleness, making sectioning difficult	
Permanganates, dichromates, or osmium tetroxide	Oxidizing agents that cross-link proteins	Fixation of cytoplasm without precipitation; fixation of lipids, especially phospholipids	Causes substantial denaturation; uncommon	
Picrates	Oxidizing agents that cross-link proteins	Good retention of nuclear structure with limited tissue hardening	Explosion hazard; causes cell shrinkage	

Tissue that is not appropriately fixed will not embed and section well, and subsequently will not stain well. Adequate fixation time is of primary importance in quality assurance. Fixatives usually are delivered at a 15:1 to 20:1 ratio of fixative volume to tissue mass. Alternatively, multiple changes of fixative can be used with agitation. It is important to expose tissues to fixatives for a sufficient time, making sure that the sample does not dry before or during this process. Even small tissue pieces may require 12–24 h of exposure time. Tissue handling also is important because areas that are physically gripped may not be fixed as rapidly as areas that are not gripped.

Typical concentrations for some fixatives are shown in *Table 1*. Note that if fixation concentrations are too high or if fixation time is too long, structural artifacts can form. The fixation temperature can be increased somewhat but not to the extent that will cause protein denaturation.

Buffers also are important for high-quality fixation. A pH range of 6–8 usually is best because it will most likely maintain the native tissue structure. Buffering must be sufficient to overcome acidification caused by hypoxia and to prevent black deposits of formalin–heme.

Tissue Processing

DEHYDRATION

Once the tissue is properly fixed, it is ready for dehydration and the embedding process. The most common dehydrants are alcohols, but sometimes acetone is used because it rapidly fixes the tissue (although it is a fire hazard). Tissues may be physically damaged if they are insufficiently dehydrated, often because of water contamination of ethanol wash solutions. After dehydration, the tissue is placed in a clearing agent before embedding. Some common clearing agents are the following: xylene, toluene, chloroform, methyl salicylate, limonene (a volatile oil found in citrus peels), or some commercial xylene substitutes.[1]

EMBEDDING

Depending on the tissue type and subsequent staining protocol, there are several embedding and mounting-media options. Paraffin is most commonly used to embed tissues. Its density is similar to that of most tissues, which facilitates sectioning, and multiple paraffin types with different melting points and hardness can be evaluated if tissue sections do not cut smoothly. If very thin sections are needed or if harder tissues (e.g., bone) will be embedded, plastics are good options (e.g., methacrylate, glycol methacrylate, araldite resin, or epon). Once the mounting medium is selected, the specimen should be carefully aligned and oriented in the medium relative to the desired cutting plane. Tissues that are properly fixed and embedded, if stored appropriately, can stay in this form indefinitely before staining.

Tissues can be embedded manually or with automated systems. Some embedding systems have a chamber for keeping molds and samples hot and ready for embedding. They also may have a separate chamber to melt paraffin, an attached dispenser for pouring paraffin into embedding molds, and a separate cold plate to cool the recently embedded tissues. Both

[1] For example, Clear Rite, Pro Par Clearant.

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manual and automated station methods are essentially the same and are well accepted as tissue embedding methods. The only difference is that the latter are semi-automated so that the system is operational within an hour or less (i.e., paraffin is melted, heating chambers are warmed, and the cold plate is cooled). Manual methods are somewhat slower. Regardless of the system used, the integrity of the histological section must not be compromised.

SECTIONING

When tissue blocks are ready for staining, sections are cut with a sharp knife or disposable blades. Paraffin-embedded tissue sections typically are 4–8 µm thick, but plastic-embedded tissues usually are 2 µm thick. The tissue sections are placed on slides and can be stored in this state. The slides are cleared again (to dissolve the paraffin) and are rehydrated before staining or IHC. In all cases analysts should be aware of the importance of each step in order to avoid producing artifacts (e.g., fixation-related shrinkage or nicks, folds, or tears in the tissue sections caused by improper microtome work or a knife that is too dull). Once the staining procedure is complete, analysts should apply sufficient mounting medium over the tissue sections in order to avoid entrapment of air bubbles when a cover slip is applied.

Example Protocol

The following is an example of a fixation, paraffin-embedding, and processing method that is commonly used before hematoxylin and eosin (H&E) staining to study the effectiveness of tissue decellularization processes.

Fixation solution: 0.1 M sodium phosphate, pH 7, containing 10% formalin

Paraffin: Melt paraffin at 60° for 3 h before analysis. [NOTE—For proper infiltration and success in later steps, it is best to keep the *Paraffin* fresh (free of clearing agent) with frequent changes of paraffin (at least two changes). The temperature of the *Paraffin* must be NMT 2°–4° above its melting point; otherwise, the tissues exposed to overheated *Paraffin* will overharden.]

Paraffin solution 1: *Paraffin* and xylene (1:3) or a xylene substitute[2]

Paraffin solution 2: *Paraffin* and xylene (1:1) or a xylene substitute

Paraffin solution 3: *Paraffin* and xylene (3:1) or a xylene substitute

Fixation and dehydration, clearing, and paraffin infiltration of the tissue (DCI): Place each tissue sample (NMT 1 cm^3) in *Fixation solution* for a minimum of 6–8 h. The volume of *Fixation solution* should be NLT 15–20 times the volume of the tissue sample. [NOTE—When processing bone samples, thoroughly fix before decalcification and DCI. In this case, after fixation, rinse the sample with Purified Water, and place in a rapid decalcifier solution[3] for the recommended time. After decalcification, rinse the bone samples with Purified Water again, and place them back in the *Fixation solution*.] Place each tissue sample in an embedding cassette, and label the cassette with a pencil or histology pen. Place cassettes in the following series of solutions for 30 min each: 2 changes of 70% ethanol, 2 changes of 95% ethanol, and 3 changes of 100% ethanol. [NOTE—An optional approach is to transfer cassettes directly from 100% ethanol to 100% xylene for 3 changes. Place cassettes in *Paraffin* heated to 2°–4° above its melting point for 1–3 changes for NLT 90 min and NMT 180 min. Exposure to prolonged heat causes shrinkage and hardening of tissues. Avoid overnight treatment.] Next, place cassettes in the following series of solutions for 60 min each at 60°: *Paraffin solution 1*, *Paraffin solution 2*, and *Paraffin solution 3*.

Tissue embedding: [NOTE—This example is a manual method, but an automated embedding method can be substituted.] Move the container with the cassettes described above from the incubator into a 60° water bath to prevent the infiltrated paraffin from solidifying during the embedding procedure. [NOTE—For easier release later from the embedding molds, molds can be sprayed with a diluted mold-release concentrate.] Place a small amount of *Paraffin* into a room-temperature base mold. Using forceps, remove a tissue-containing cassette from the liquid *Paraffin*, and position the tissue section with the desired orientation in the mold before the *Paraffin* solidifies. [NOTE—If it is not positioned properly, the tissue sample can be placed back into the tissue cassette containing the liquid *Paraffin*, and then a new mold can be prepared and tissue positioning can be attempted again.] Once the tissue is in place, place the labeled portion of the tissue cassette onto the base mold, and add *Paraffin* to a level just below the lip of the cassette. Place the cassette mold assembly onto a frozen cold plate. When the *Paraffin* begins to solidify on top, transfer the assembly to an ice bath until the entire block is solidified (about 20 min). Once the *Paraffin* is solidified, carefully separate the base mold from embedded tissue without using excessive force. Store blocks at 4° until needed for sectioning. Blocks can be sectioned immediately, but it is advisable to wait at least 24 h for best results.

Sectioning paraffin-embedded tissue: Using an appropriate marker, label glass slides with a tissue-sample identifier. Select an embedded tissue block, and trim any excess wax remaining around the edges of the block. Fill a flotation bath with Purified Water, with or without gelatin (histology samples only), and warm to 36°–46°. Carefully place a new microtome blade into the blade holder. Ensure that the blade is locked into place. If available, select the manual or motorized mode of the microtome. Place the tissue block in the microtome cassette holder, and adjust its orientation using the adjusting screws until the block is correctly positioned vertically and horizontally in relation to the blade. Select the *trim* setting. [NOTE—The trim cut usually is set to 15–16 µm, but this can be increased if a large amount of trimming is necessary, or can be decreased if only a small amount of tissue is present in the block. In addition, blocks can be refrigerated or placed on ice before sectioning to keep them cool and firm.] Advance the block forward to the desired position. Once the block is positioned, start trimming or "facing" the block. After the tissue is visible in the sections, set an appropriate sectioning depth and begin collecting tissue sections. [NOTE—Routine H&E sections should be cut at approximately 6 µm, but the section thickness can be adjusted between 4 and 8 µm as necessary if there is difficulty obtaining good-quality sections.] Using forceps, transfer the sections to the tissue flotation bath. Gently place the ribbon of sections onto the water surface while gently pulling to eliminate any wrinkles from the ribbon. [NOTE—If the water in the flotation bath becomes too hot, the tissue sections will fragment. If this occurs, decrease the heat setting on the bath, and allow the water to cool before proceeding.] Next, separate one or two adjacent, intact, full sections from the ribbon with a sharp dissecting probe or forceps. Using a glass slide placed at approximately a 45° angle beneath the section, lift the sections onto the slide. Gently tap off the excess water, and allow the sample to dry. As

[2] For example, CitriSolv, Clear Rite 3, Pro Par Clearant, or equivalent.
[3] For example, Decalcifying Solution, Richard-Allen.

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needed, repeat the sectioning process for additional samples. After all slides have been prepared and before staining, allow the slides to air-dry for NLT 15 min or until no water droplets are visible. Remove the wax blocks from the microtome, and store appropriately.

Deparaffinization and rehydration of histological slides: Before staining, place slides with mounted tissue specimens in either Coplin jars, reagent buckets, or staining racks. Place slides in the following solutions for at least 3 min each: 3 changes of xylene (or a xylene substitute), then 100% ethanol, then 95% ethanol, then 70% ethanol, then 2 changes of Purified Water. Proceed with the desired staining procedure. [NOTE—Slides can be left in the water for several hours but then must be stained.]

METHOD DEVELOPMENT AND VALIDATION: POINTS TO CONSIDER

Histological methods sometimes are used to demonstrate that cells within a tissue sample are still viable (e.g., when evaluating a cryopreservation method's performance). In other circumstances, the method is used to evaluate the absence of viable cells following a decellularization process. Each case presents specific validation challenges.

H&E staining provides a useful tool for determining whether significant quantities of cells are still viable after preservation. During method validation, immunohistological stains can be used to ensure that specific cell types are still present in the preserved samples. Testing samples for all expected cell types during the validation phase provides support for the less specific H&E staining (that will stain all cells and not just specific cell types) during routine monitoring.

When validating any histological method, analysts should demonstrate that different observers can detect the same quality (or quantity) of an object, tissue type, cell body, nucleus, etc., and that the characterization by different observers can be replicated in several specimens following the same process. Samples for evaluation may consist of multiple types of cells, extracellular matrix proteins, and glycosaminoglycans, or other components. Some of these components may be resistant to decellularization processes or may be difficult to stain. During assessment of method reproducibility, analysts should evaluate multiple samples throughout the sample to ensure that they are representative of the types of materials expected to be found in the matrix. In addition, because inadequate decellularization procedures may not remove all cells throughout the material, it is important to take samples from multiple locations in the tissue so that remaining cells are not underestimated. Intra-operator reproducibility also should be a part of the assessment because the relatively weaker signal from fewer positively stained cells may make visualization more challenging for some technicians. The method validation should use samples that underwent worst-case decellularization processing conditions as determined by statistical modeling to ensure that residual cells can still be identified and quantified in these marginal samples. Finally, the validation of the method should include control samples to confirm the suitability of the staining system (see *Hematoxylin and Eosin Staining of Sectioned Tissue for Microscopic Examination* ⟨1285.1⟩).

The histological assessment of decellularized tissue-based matrix products is one tool to characterize the matrix but cannot be the sole indicator of product quality. On the one hand, processing may generate decellularized matrix products that have no detectable cells but may not possess other critical quality attributes. On the other hand, tissue matrix products undergoing other decellularization processes may result in products with greatly reduced cellular content (although some cells may remain) that retain the necessary performance characteristics for that therapeutic purpose.

Sample Selection for the Analysis of a Decellularization Process

Samples cannot always be taken from a random location within the product. For example, it is not practical to pull a sample from the center of a sheet of decellularized pericardium without creating a defect that would make the end product undesirable. If routine sampling plans require sampling from the edges or waste portion of a given matrix, analysts should conduct an initial validation study that confirms that such a sample is representative of the whole. This is important because the geometry of the matrix may affect the exposure of the matrix to the decellularization processing solutions. For example, if the tissue matrix is tubular, e.g., a nerve segment, and a sample is taken from the end for histological assessment, then this sample will have different exposure to solutions than a sample taken from the center of the nerve segment. Thus, the sampling may not represent a worst-case assessment, and the suitability of the sample should be determined by means of a validation study.

CONCLUSIONS

This chapter outlines common steps to prepare tissue or organ specimens for light microscopic analysis following either staining or treatment with structure-specific probes. Each of these steps can produce specimen artifacts that either interfere with the observation or actually introduce structures that were not present in the original sample (e.g., certain well-known chemical artifacts). Therefore, proper attention and consistent execution of each step are critical factors in generating sections that reflect the character of the tissue and that stain in a reproducible manner.

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⟨1285.1⟩ HEMATOXYLIN AND EOSIN STAINING OF SECTIONED TISSUE FOR MICROSCOPIC EXAMINATION

INTRODUCTION

Histologic methods involve the preparation of organs, tissues, or cells for microscopic analysis of constituent elements that have been exposed to particular chemistries or immunochemical staining procedures. Histologic methods have many goals, and are often used to evaluate native and processed therapeutic tissue-based products. For example, these methods can be used to establish the integrity of the tissue with regard to cellular content and extracellular constituents. Nuclear staining intensity and nuclear shape may be altered if tissue recovery or handling is compromised. Furthermore, extracellular structural and accessory components such as collagen, elastin, and glycosaminoglycans can be specifically stained so their presence and location can be determined.

Histologic methods are particularly useful for detecting trends in content (e.g., cell numbers and amounts of tissue constituents). Therefore, they have been valuable in the development and verification of tissue-processing methods intended to reduce or eliminate cellular content (decellularization detected by reduced nuclear staining, for example with hematoxylin) while maintaining extracellular matrix structure and components (e.g., retention of glycosaminoglycans or other carbohydrates assessed by persistence in Alcian blue staining).

Tissue histologic analysis typically begins with a routine staining procedure using hematoxylin and eosin (H&E) to detect cellular chromatin and cytoplasm and extracellular structures (e.g., collagen and muscle). Hematoxylin acts as a basic dye. In a metal complex it binds to acidic structures such as cellular nucleic acids (DNA or RNA), producing the blue–purple stain typically noted as nuclear staining. Additionally, in calcified tissues it forms a blue or purple precipitate. Eosin acts as an acid dye and stains basic materials red (muscle) to pink (collagen). Eosin staining is responsible for the detection of the cytoplasm and intracellular and extracellular proteins, but it does not allow identification of specific intracellular structures or proteins. H&E does not stain fat itself, but fatty areas can be identified by the staining of the stroma, yielding an outline of adipocytes.

H&E staining is often used to demonstrate the reduction of nuclei following tissue decellularization processes, but H&E staining also provides structural information. H&E staining reveals that Type I collagen fibrils have a particular arrangement and wave pattern depending on the tissue observed and the orientation of the specimen. Altered collagen fibril patterns, e.g., smearing and loss of fibrils, may suggest disruption of the native structure of the tissue. Decellularization methods, which can alter the structural integrity of tissue, may reduce the staining intensity of collagen. Alternatively, the staining intensity can be artificially increased if residual charged processing chemicals remain in the tissues. H&E staining can help identify noncollagen protein structures. For example, elastin fibers are not specifically stained by this method, but thick elastin fibers such as the internal elastic lamina of arteries are easily detected by their profound waviness and subintimal location shown by staining. A mineralized matrix (e.g., in bone) can be seen as dark red to purple (depending on the hematoxylin choice) granular staining within fields of structural protein fibers.

H&E staining is a starting point for more detailed histologic analysis. Differential staining of collagen (typically colored blue) is achieved with Masson Trichrome stain, and Verhoeff's Van Gieson and Movat Pentachrome stains highlight elastin (black). The other major components of connective tissues are the glycosaminoglycans, and these can be detected and to some extent differentiated with various cationic dyes such as Alcian blue in Movat's Pentachrome stain, for example.

More detailed compositional analysis of tissues and observations of the effects of decellularization methods require structure-specific probes. At the level of light microscopy, this specificity is provided by immunohistochemical staining using antibodies to targeted structural proteins and extracellular matrix constituents or by using labeled lectin probes to detect certain sugar structures. The latter are of particular utility for evaluating the removal of important xeno-antigenic epitopes.

The purpose of this chapter is to identify the factors that should be controlled to optimize the consistency of the results of tissue staining with H&E. In addition, the chapter provides a common H&E staining procedure.

BASIC PRINCIPLES

Hematoxylin is oxidized to hematein, which complexes with a metal cation, binds to negatively charged groups, and stains basophilic substances such as nucleic acids. The most common cation for this application is aluminum (III), but iron (III) and tungsten also are important. Hematoxylin also stains calcium deposits. The most common metal used in hematoxylin staining is aluminum, and the resulting stain produces a blue color. This aluminum salt form of hematoxylin requires a pH >5 to form an insoluble blue aluminum hematein complex. The high pH is obtained in a bluing step using a weak alkali solution such as ammonium water. Because anionic materials often are used to process tissue (e.g., anionic detergents), these materials should be removed from the tissue first, or nonspecific hematoxylin staining may be seen.

Eosin is an acidic analine dye with affinity for cationic amino acids such as arginine and lysine in proteins. Cytoplasm, muscle, connective tissue, colloid, red blood cells, and decalcified bone matrix all stain pink to pink/orange/red with eosin. Eosin methods use solutions containing various proportions of water, ethanol, and acetic acid. The uptake of eosin in cells is promoted by higher eosin concentration, a higher proportion of water than ethanol, and the inclusion of acetic acid.

PROCEDURES—POINTS TO CONSIDER

It is important to control the consistency and quality of the stain reagents and process. Each new lot of stains should be qualified with known control specimens to ensure consistent staining processes. Solution color should be monitored, and if colorless solutions become colored they should be discarded (especially the alcohols and xylene). Staining solutions can age,

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and their staining effectiveness changes over time. Thus analysts should establish or assign a shelf life to critical staining reagents. Some reagents form precipitates or undergo overt color changes over time. Best practices indicate that such solutions should be replaced. Labeled expiration dates of commercial stains should be followed closely. If reagents are mixed in the laboratory, analysts should monitor their performance over time using control specimens to assess staining intensity and differentiation. Expiration dates should be added for each laboratory reagent. Best practices require that to the extent possible samples to be compared should be stained at the same time. To facilitate comparison, staining should be monitored and corrected if necessary. Regressive staining can be performed if overstaining with hematoxylin occurs. In this case, excess stain is removed with acid (e.g., 0.5% HCl in 70% ethanol) in a step called differentiation. Excessive hematoxylin staining can block eosin staining. Ideally, progressive staining, or real-time monitoring of the staining process to the desired intensity, should occur.

As with any tissue-staining method, H&E staining intensity may be affected by steps taken before the staining procedure, the chemicals used, and the conduct of the staining itself. Section thickness can affect the overall specimen depth and can influence the apparent stain. Inadequate removal of paraffin wax can prevent interaction of the aqueous solutions of the staining steps with the tissue. The color can be too strong if certain hematoxylin reagents are not diluted before use, if the stain is applied for too long, if the differentiation step is too short, or if the acid is too dilute. Weak staining can be the result of exhausted hematoxylin, short staining time, overdifferentiation, or low pH during the bluing step.

SYSTEM SUITABILITY

Control tissue should be used during each run to ensure that all the reagents are working appropriately and the processing regime does not adversely affect staining. Untreated fresh tissue makes a good positive control, because there are no processing reagents that could affect the staining process. An appropriate negative control is an unstained sample from a previous lot that met all the product quality criteria of the H&E analysis. Controls are important because processing methodologies such as decellularization or cross-linking may positively or negatively affect the ability of the reagents to stain the tissue as expected and can contribute to lot-to-lot variability in staining intensity.

QUANTITATION

A physical count of positively stained cells in a given area (cells/mm^2) is preferable to simply comparing a sample to a previously submitted visual reference, particularly for products that undergo a decellularization process that yields a greatly reduced (yet nonzero) number of positively stained cells. Many, though not all, samples of tissues that have undergone a process of cellular removal still have identifiable cellular debris after processing. It is difficult to compare these nonzero samples to a visual reference, so a standardized count per area is a better comparison of process effectiveness.

Software-based automated cell-counting systems that are available on many microscopy systems must be validated to confirm that the software appropriately counts cells and sample area. When the tissue has not been decellularized, there should be a high value of positive signals, and the system should be able to accurately differentiate that tissue from other samples with low cell counts. Validation is important when analysts attempt to quantify the number of cells in a given area on irregularly shaped samples because the algorithm used to estimate areas may have limitations when applied to these types of samples. Sample area should be measured accurately when there is a low cell count, because a large error in the denominator can cause a significant error in the reported cell density.

EXAMPLE PROTOCOL

A common H&E method is described below. Analysts can follow this example after preparing and deparaffinizing tissue sample slides as described in *Preparation of Biological Specimens for Histologic and Immunohistochemical Analysis* ⟨1285⟩. This protocol is commonly used to study the effectiveness of tissue decellularization.

Bluing reagent: 3 mL of 28% ammonium hydroxide in 1 L of *Purified Water*

Staining method: After deparaffinizing tissue-containing slides, place the slides in a hematoxylin solution[1] for 1.5–3 min. [NOTE—Staining times can vary depending on hematoxylin brands (and type) and the tissues being stained, so optimization of staining time is required in this step. For this reason, laboratories should track the reagent sources to ensure consistency.] Rinse slides under running tap water for 0.5–3 min. If necessary, perform a differentiation step to remove excess stain so the desired element or structure is left stained by placing the slide in a clarifying solution[2] for 30–60 s followed by rinsing in tap water for 30–60 s. Next, place slides in *Bluing reagent* for 1–2 min. Dip slides 12 times in *Purified Water*. Rinse thoroughly in tap water for 0.5–2 min to remove the *Bluing reagent*. [NOTE—Insufficient rinsing affects subsequent staining because of a change in pH.] Next, place the slides in the following solutions for the specified times: 95% ethanol for 30 s to 1 min [NOTE—The time will vary depending on eosin type and components.] then place the slides in eosin solution[3] for 45 s to 3 min, 70% ethanol for 3 min, 95% ethanol for 3 min, and finally 100% ethanol for 3 min. [NOTE—Alcohol washing must be closely monitored to avoid excessive removal of eosin stain. Excessive removal of eosin from the extracellular matrix can be seen as pale pink staining.] Place slides in three changes of fresh xylene or xylene substitute for 1–3 min each. Place a couple drops of mounting medium on the slide, and apply the cover slip.

Expected results: Nuclei should be stained blue. Cartilage and calcium deposits should stain various shades of blue. Cytoplasm and other tissue constituents stain various shades of pink. Erythrocytes and eosinophilic granules stain bright pink to red.

[1] Richard Allan Scientific #7231 or suitable equivalent.
[2] Clarifier 2 from Richard Allan Scientific or suitable equivalent.
[3] Richard Allan Scientific #7111 or suitable equivalent.

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CONCLUSIONS

It is critically important to test and verify staining conditions for each new tissue being studied. Staining intensity may be altered by tissue treatment before processing. A notable example is the treatment of tissues to decellularize or antigenically modify them, because these procedures may affect tissue components and may alter the staining of the remaining constituents.

Add the following:

▲⟨1430⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—GENERAL

1. OVERVIEW: GENERAL CHAPTERS BASED ON SCATTERING PHENOMENA
2. INTRODUCTION
3. THEORY (GENERAL PRINCIPLES OF SCATTERING)

1. OVERVIEW: GENERAL CHAPTERS BASED ON SCATTERING PHENOMENA

This chapter provides a general overview of the scientific principles and analytical procedures used in scattering techniques and their applications. *Figure 1* shows a taxonomy (tree diagram) of the techniques that are covered in this chapter. The first branch classifies the techniques according to the wavelength of the incident electromagnetic (EM) radiation: visible light (700–400 nm), X-rays (0.33–0.06 nm), as well as neutrons (2.5–0.15 nm). The next branch classifies techniques into those based on the measured intensities (i.e., scattering), where the radiation exiting the sample is measured at an angle relative to the incident beam, or transmission, where this angle is equal to zero. Scattering techniques can be further classified based on how the exiting radiation is quantified, in either a time-averaged (static) or time-dependent (dynamic) mode. Finally, because scattering techniques are often coupled with separation techniques, such as chromatography or field flow fractionation, these are included as well.

Two of the most important parameters of the EM radiation–matter interaction are the wavelength of the incident EM radiation ($\lambda = \lambda_0/m_0$, where λ_0 is the wavelength in vacuo and m_0 is the refractive index of the medium) and the size of the particle, often expressed as an equivalent spherical radius (r). A dimensionless parameter ($\alpha = 2\pi r/\lambda$) can be used to describe different scattering regimes. For $\alpha \ll 1$, the scattering is considered as being in the Rayleigh regime and the Rayleigh scattering theory applies (named after Lord Rayleigh, who developed the theory in 1871); for $\alpha \sim 1$, the scattering is considered as being in the Mie regime and the Mie scattering theory applies (named after Gustav Mie, who developed the theory in 1908); and for $\alpha \gg 1$, geometric optics apply. These theories are further addressed in the respective chapters where they are applied. *Table 1* lists all chapters whose fundamental physical principles are addressed in this overarching chapter. These chapters deal with elastic light scattering in heterogeneous systems and its applications in the pharmaceutical industry.

Table 1. List of ⟨1430.X⟩ General Chapters Family

Chapter Number	Chapter Title	Measured Property	Primary Purpose
⟨1430⟩	*Analytical Methodologies Based on Scattering Phenomena—General*	Overarching chapter	General overview; N/A
⟨1430.1⟩	*Analytical Methodologies Based on Scattering Phenomena—Static Light Scattering*	Scattered light intensity as a function of detector's angle	Molecular weight, size, shape; molecular interactions
⟨1430.2⟩	*Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size*	Diffracted light intensity at multiple angles	Particle size distributions in the approximate range of 0.01–3000 µm
⟨1430.3⟩	*Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering*	Fluctuations of the scattered light intensity	Average hydrodynamic diameter and polydispersity index
⟨1430.4⟩	*Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)*	Doppler shift of scattered light frequency	Stability of suspensions and emulsions
⟨1430.5⟩	*Analytical Methodologies Based on Scattering Phenomena—Small-Angle X-Ray Scattering and Small-Angle Neutron Scattering*	Scattered intensity of a beam of X-rays or neutrons	Direct probing of the size, shape, ordering, and interactions of individual molecules, their assemblies in the approximate length scales from 0.1–2500 nm for SAXS and 0.2–1000+ nm for SANS. Also properties of condensated phrases (e.g., porosity and crystallinity) can be determined.

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Table 1. List of ⟨1430.X⟩ General Chapters Family *(continued)*

Chapter Number	Chapter Title	Measured Property	Primary Purpose
⟨1430.6⟩[a]	*Analytical Methodologies Based on Scattering Phenomena—Particle Counting via Light Scattering*	Light scattered from individual particles passing through a light beam	Quantitation of particles in liquids and gases from 0.1–2 µm
⟨1430.7⟩[a]	*Analytical Methodologies Based on Scattering Phenomena—Nephelometry and Turbidimetry*	Direct or indirect measurement of the scattered light intensities	Suspended particles in liquid or gas samples; nephelometry for lower aggregate sizes and analyte concentrations; turbidimetry for higher aggregate sizes and analyte concentrations

[a] This chapter will be published in a future *Pharmacopeial Forum (PF)* issue.

Because these methods are based on the same underlying physics, the general principles are given only once in this overarching chapter. The relationships between these methods are shown in *Figure 1*.

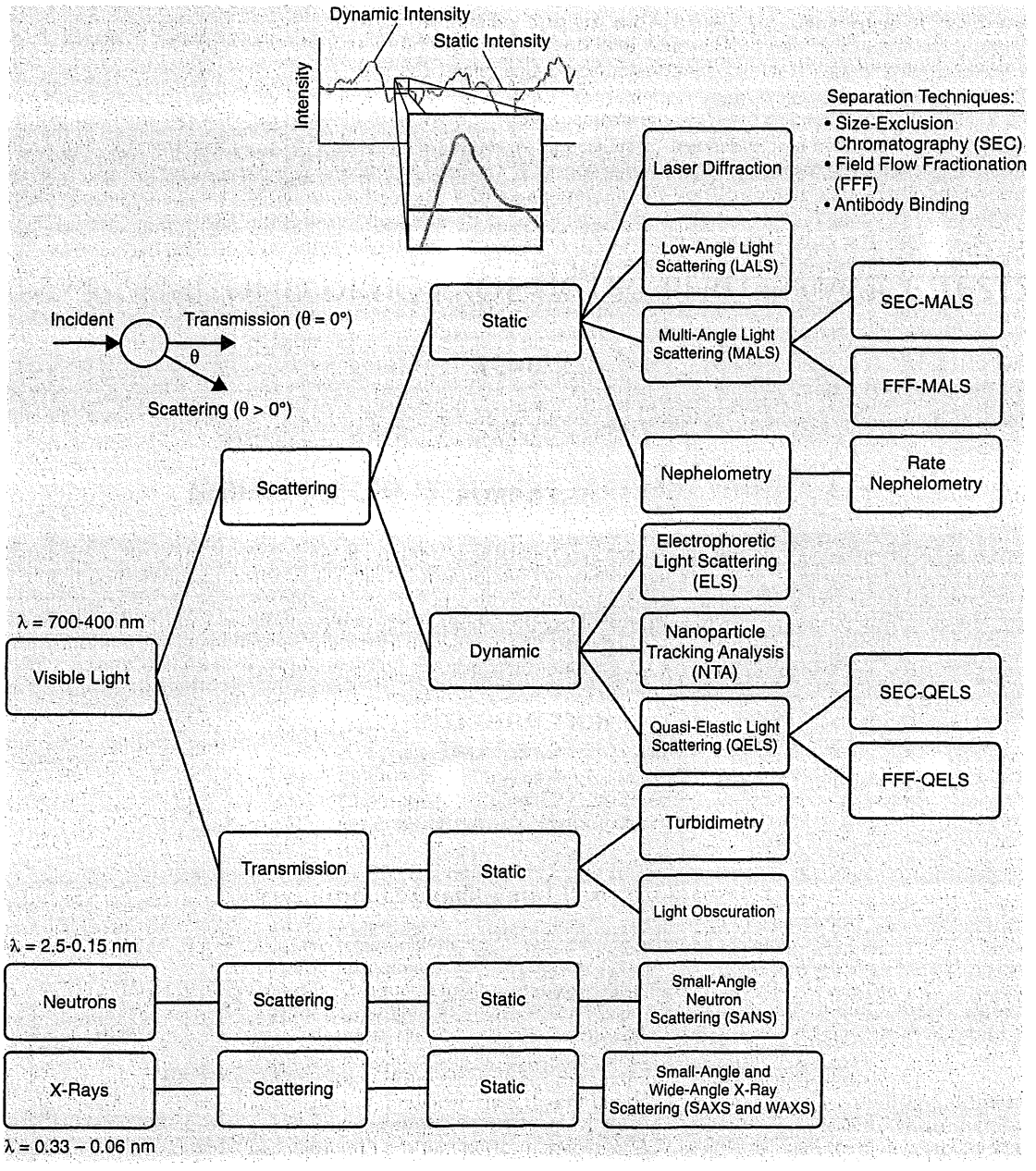

Figure 1. Taxonomy of scattering methods.

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2. INTRODUCTION

When an EM wave strikes/interacts with a small object (a particle or a molecule) and thereby changes its direction, the phenomenon is called scattering. If the scattered EM radiation has exactly the same energy (wavelength) as the incident one, it is called elastic scattering. When the energy (wavelength) of the scattered EM radiation is different from that of the incident EM radiation, the scattering process is termed "inelastic". Inelastic scattering is exploited, as an example, by Raman spectroscopy (refer to *Raman Spectroscopy* ⟨1120⟩). Scattered radiation may be detected and measured either directly: as a function of the angle between the incident beam direction and the detector, or indirectly: when the actual measurement is that of the transmitted light. The latter is the case for both turbidity and light obscuration methods (see the following chapters on turbidity and light obscuration: *Nephelometry, Turbidimetry, and Visual Comparison* ⟨855⟩, *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, *Particulate Matter in Injections* ⟨788⟩, and *Methods for the Determination of Particulate Matter in Injections and Ophthalmic Solutions* ⟨1788⟩). Both methodologies exploit time averaged signals.

In static light scattering (SLS), the inevitable short-term temporal fluctuations in scattering intensity due to Brownian motion are averaged over a range of times from tens to hundreds of milliseconds. SLS therefore measures the "time average" intensity of scattered light from particles in a (suitably prepared) sample. Depending on the specific technique, SLS provides information about molecular weight, particle size, particle shape, and molecular interactions (see *Analytical Methodologies Based on Scattering Phenomena—Static Light Scattering* ⟨1430.1⟩. Light scattered in the near-forward direction by particles is analogous to diffraction of light through an aperture. This is exploited by (laser) diffraction techniques, which are optimized to afford the derivation of a full size distribution, with moderate to high resolution, rather than a single characteristic size as in low-angle light scattering (LALS)/multi-angle light scattering (MALS).

Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) are also static techniques in that they make use of time averaged signals. These differ from LALS (and MALS) in that they are based on scattering of X-rays (SAXS) or neutrons (SANS) rather than visible light. Like SLS, these techniques also measure size, shape, and interactions but on much shorter length scales, typically ranging from around 1 nm to several hundred nanometers. Very/ultra small-angle (VSAXS/VSANS, USAXS/USANS) and wide-angle (WAXS/WANS) analogues extend these length scales to the micrometer and sub-nanometer regimes, respectively. These applications are discussed in *Analytical Methodologies Based on Scattering Phenomena—Small-Angle X-Ray Scattering and Small-Angle Neutron Scattering* ⟨1430.5⟩.

Dynamic light scattering (DLS), which is addressed in *Particle Size Analysis by Dynamic Light Scattering* ⟨430⟩[1] and *Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering* ⟨1430.3⟩, differs from SLS in that DLS measures the fluctuation of the scattered light intensity over very short time intervals (e.g., approximately 200, 400 ns).

Electrophoretic light scattering (determination of zeta potential), which is addressed in more detail in *Determination of Zeta Potential by Electrophoretic Light Scattering* ⟨432⟩[1] and *Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)* ⟨1430.4⟩, measures the Doppler shift of the frequency of scattered light as the result of particle movements from the cumulative effect of electrophoresis and electroosmosis.

3. THEORY (GENERAL PRINCIPLES OF SCATTERING)

Light/EM scattering is the result of a complex interaction between incident light/EM waves and matter. An EM wave that is redirected, i.e. changes direction, when it encounters obstacles such as a molecule or molecular aggregates, or particles, is said to have been scattered. As the EM radiation, such as light or X-rays, interacts with a discrete particle, the electron orbits within the particle's constituent atoms or molecules are perturbed periodically with the same frequency (ν_0) as the electric field of the incident wave. The oscillation or perturbation of the electron cloud results in a periodic separation of charge within the molecule, which is called an induced dipole moment. The oscillating induced dipole moment manifests itself as a source of EM radiation (see *Figure 2*). Neutrons, on the other hand, are always scattered by a very short-range nuclear interaction with the nuclei in the atoms.

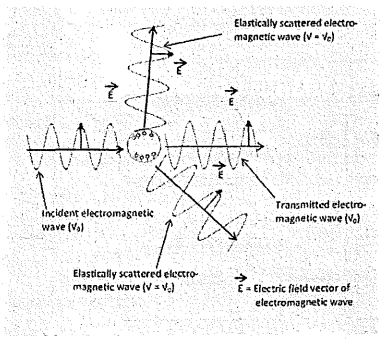

Figure 2. Scattering of an incident EM wave by an induced dipole moment.

The (time averaged) intensity (I) of the light beam as it interacts with matter along its path (i.e., when the incident light is detected at an angle of zero) decreases exponentially with the thickness, x, of the layer of material as follows in *Equation 1*:

[1] This chapter will appear in a future *Pharmacopeial Forum* (*PF*) issue.

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$$I = I_0 e^{-\tau x} \quad (1)$$

where τ is the turbidity and x is the distance that the incident light travels through the sample.

This equation is the basis for turbidimetry and nephelometry (see ⟨855⟩).

In SLS, the theoretical description of the relationship between the (time averaged) intensities of incident and scattered light, although quite complex, originates from the following simple relationship (*Equation 2*).[2]

$$I = I_0 \frac{1}{r^2} \sigma_{scat} \quad (2)$$

The scattering coefficient, σ_{scat}, and with it the intensity of the scattered light depend on factors determined by the interaction of the incident light with the material/particles itself as well as those stemming from the equipment used for generating and detecting the signal (e.g., r, the distance of the detector from the sample; I_0, the intensity of the incident light; and, Θ, the angle of observation).

On the other hand, when studying the fluctuations in scattered intensity (DLS), the way to extract useful, quantitative information is by calculating the so-called "autocorrelation function", denoted $g_2(\tau)$ based on the measured intensities (*Equation 3*):

$$g_{2,theory}(\tau) = (\Sigma A_i e^{B_i \tau})^2 + 1 \quad (3)$$

where in this case, τ denotes a correlation time (not to be confused with the τ in *Equation 1* above), and the use of the index, i, accounts for different particle types (e.g., different sizes) present. A computer program is used to find the parameters A_i, B_i (= A_1, B_1, A_2, B_2,...) that produce the best agreement between the measured and theoretical autocorrelation function.

Depending on the methodology, the equations above are transformed to obtain a measure that relates the measured intensities, I and I_0, respectively, and the autocorrelation function $g_2(\tau)$ to the material itself, independent of equipment characteristics. These are further discussed in the specific chapters in the *USP–NF* dedicated to the corresponding techniques (see *Table 1*).▲ (USP 1-Dec-2019)

Add the following:

▲⟨1430.1⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—STATIC LIGHT SCATTERING

1. INTRODUCTION
2. THEORY
3. APPLICATIONS
   3.1 Low-Angle Light Scattering Determination of Molecular Weight
   3.2 Multi-Angle Light Scattering
   3.3 Combination of Light Scattering with Size-Fractionation Techniques
4. INSTRUMENTATION
5. LOW-ANGLE LIGHT SCATTERING VERSUS MULTI-ANGLE LIGHT SCATTERING
6. PRACTICAL CONSIDERATIONS
7. ADDITIONAL SOURCES OF INFORMATION
REFERENCES

1. INTRODUCTION

The phenomena observed when photons [i.e., an electromagnetic (EM) wave] strike or interact with a small object (a particle or a molecule) and thereby change direction is called light scattering (LS).

In static light scattering (SLS), the inevitable short-term temporal fluctuations in scattering intensity due to Brownian motion are averaged over a range of time scales from several hundredths to several tenths of a second. SLS measures the time average intensity of scattered light from particles in a suitably prepared sample; the detector signal is, therefore, time independent or "static".

In SLS, the scattered light is detected and measured as a function of the angle between the detector and the incident beam direction. Light scattering can be either at a single fixed angle, as in low-angle light scattering (LALS) or right-angle light scattering (RALS); or over a range of angles, as in multi-angle light scattering (MALS).

SLS provides information about molecular weight, particle size, particle shape, and molecular interactions.

This chapter provides guidance and procedures for LALS and MALS. These SLS methodologies are based on the Rayleigh approximation of classical Mie scattering theory. In these scattering methods, the particles are assumed to be present in solution

[2] Hahn DW. *Light Scattering Theory*. University of Florida; 2009. http://plaza.ufl.edu/dwhahn/Rayleigh%20and%20Mie%20Light%20Scattering.pdf.

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(or gas) and free to move; thus, the particles are inherently in an unordered state. Other scattering phenomena such as reflection, refraction, and diffraction require the scattering particles to be highly ordered and are out of the scope of this text.

2. THEORY

SLS techniques are based on measurement(s) of the scattered light intensity (I_S) itself, as opposed to indirect measurements (e.g., inferring the scattered intensity from the attenuation of transmitted light).

In order to obtain a measure that relates the material itself to the scattering intensity, independent of equipment characteristics, one defines the Rayleigh ratio, $R(\Theta)$, for the sample under investigation (*Equation 1*):

$$R(\theta) = \frac{I_S(\theta) r^2}{I_0 V_S(\theta)} \qquad (1)$$

$R(\Theta)$ is defined as the scattered intensity per unit solid angle, scattering volume (V_S), and incident intensity (I_o) that is in excess of that scattered by solvent alone, and r is the distance of the scattering volume from the detector.

Figure 1 shows that the scattering of light on the particle changes the wave vector from $k_{incident}$ to ~$k_{scatter}$. For sufficiently small particles, only the direction, but not the wavelength, changes as a result of the collision. Hence, the length of the wave vector $\vec{k}$, remains unchanged.

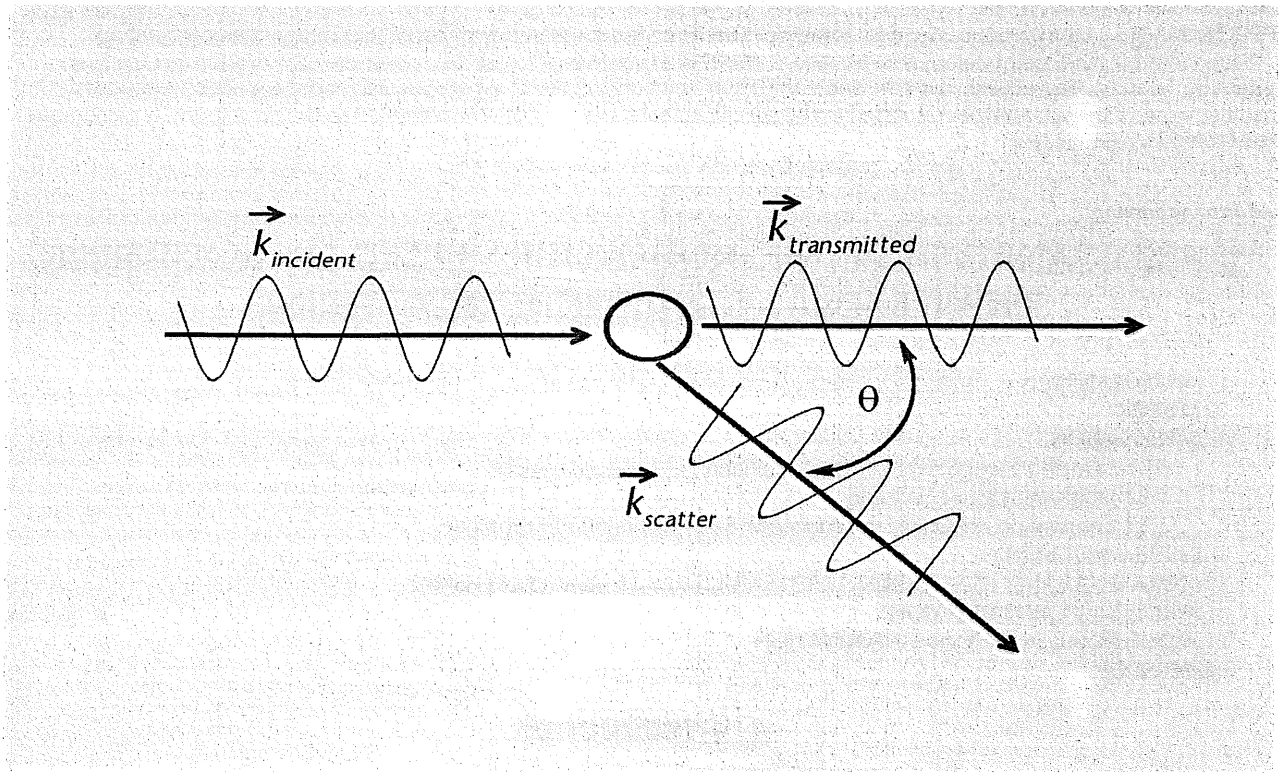

Figure 1. Scattering of light at an angle Θ.

As the EM wave interacts with the electron orbits of the constituent molecules, the orbits are perturbed periodically at the same frequency as the electric field of the EM wave. The oscillations of bound and free charges in turn generate EM waves inside and outside of the particle.

Although Maxwell's equations for an EM light wave interacting with a sphere completely describe these interactions, proper boundary conditions for electric and magnetic fields are needed to find the exact solutions for these equations, along with the values of the quantities of interest. Moreover, such solutions are difficult to find.

One of the rare exact solutions for the scattering of a plane wave by a uniform sphere is obtained from the Mie theory of scattering (*1*). Mie theory has no size limitations; it converges to the limit of geometric optics for large particles. Mie theory, therefore, may be used to describe most spherical particle scattering systems. However, difficulties in finding exact solutions for the wave equations have led to the development of approximate methods of solving the scattering problem. One class of these approximations, known as Rayleigh scattering theory, describes light scattering by particles that are small compared to the wavelength of the light (*2*, *3*). Due to the complexity of the Mie scattering solution, the Rayleigh scattering theory is generally preferred, if applicable.

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When the scattering particles are much smaller than the wavelength of the light (approximately 1/20 of the wavelength or less), they are considered to be at the Rayleigh scattering regime. The EM field acting on the particle in the Rayleigh scattering regime is effectively homogenous. This is a very important approximation for biomedical optics because many of the structures from which cell organelles are built (e.g., the tubules of the endoplasmic reticulum, cisternae of the Golgi apparatus) fall into this category.

Therefore, the time for penetration of the electric field is much shorter than the period of oscillation of the EM wave. The particle behaves like a dipole and therefore radiates (scatters) light isotropically, i.e., at an equal intensity in all directions relative to the direction of the incident light. With larger particles and/or shorter wavelengths, the scattered light begins to exhibit additional interference effects from other atoms within the molecule.

In addition, the scattered light has the same wavelength as the incoming light, a process referred to as elastic scattering. Moreover, the intensity of the scattered light is independent of the particle size.

At the Rayleigh limit, the Rayleigh ratio is transformed to *Equation 2*:

$$\frac{K^* \times c}{R(\theta)} = \frac{1}{M_w \times P(\theta)} + 2A_2 \times c \qquad (2)$$

The variables are defined as follows:

$R(\Theta)$ = Rayleigh ratio

K^* = optical contrast parameter = $[4\pi^2 n_0^2 (dn/dc)^2]/(\lambda_0^4 N_A)$ and $K^* = K \times (dn/dc)^2$ where K is a constant, $K = \frac{4\pi^2 n_0^2}{\lambda_0^4 N_A}$

[NOTE—$K^* \neq 0$ only when $n \neq n_0$, i.e., $dn/dc \neq 0$. Modern instruments rely on laser light, and the incident light is polarized. For unpolarized light, the constant factor 4 is replaced with 2.]

$P(\Theta)$ = form factor = $1 + (16\pi^2/3\lambda^2)(r_g^2) \sin^2(\theta/2)$ captures the decrease in scattered intensity resulting from interference between individual scattering centers within a large molecule. At a small Θ, $P(\Theta) \approx 1$, because $\sin^2(\theta/2) \rightarrow 0$.

A_2 = second virial coefficient; describes interactions between molecules. In an ideal system/solution, $A_2 = 0$ (i.e., there are no interactions between molecules) and the intensity of scattered light will increase linearly with the concentration. Because interactions between molecules become rarer at lower concentrations (c), at the limit $c \rightarrow 0$ it is reasonable to assume that $A_2 = 0$.

M_w = weight-average molecular weight of the analyte/solute

r_g = radius of gyration, the equivalent spherical particle radius. At the Rayleigh limit, $r_g << \lambda_0$, for example in the case of visible light in which $\lambda_0 > 400$ nm, then $r_g < 15$–20 nm. This is true for folded proteins with $M_w < 5 \times 10^7$.

c = analyte concentration

λ = relative scattering wavelength: $\lambda = \lambda_0/n$

λ_0 = incident wavelength

n, n_0 = real refractive indices of the particle and solvent [refractive index (RI) in the absence of absorption]

dn/dc = the differential RI (concentration) increment, i.e., the change of n with the analyte concentration, needs to be known in advance. It should be determined separately, either from literature data or experimentally. It has a constant value for many proteins in water-based buffers and for polysaccharides:

- Proteins = 0.18–0.20 mL/g
- Polysaccharides = 0.14–0.15 mL/g

For glycosylated proteins, dn/dc may not be the same for all glycoforms.

N_A = Avogadro constant

3. APPLICATIONS

3.1 Low-Angle Light Scattering Determination of Molecular Weight

Depending on the experimental conditions, it is possible to further simplify *Equation 2*, thereby making it amenable to practical applications. In particular, at small scattering angles, $\Theta \rightarrow 0$, $P(\Theta) \rightarrow 1$. Therefore, at small angles, scattering is independent of Θ. This observation forms the basis of LALS. Note that measurements at $\Theta = 0$ are impractical because the incident (laser) light would interfere with the measurement.

3.1.1 MEASUREMENTS AT LOW CONCENTRATION

Further, at the limit of low concentrations, the term $A_2 \times c$ approaches 0. *Equation 2* then simplifies to *Equation 3*, as shown below:

$$R_{LS}(\theta) = K_{LS} \times c \times M_w \times (dn/dc)^2 \qquad (3)$$

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A prerequisite prior to applying the above equation is an accurate determination of the value of *dn/dc*, which is dependent on both the solvent and the sample/analyte. The value of *dn/dc* can be determined experimentally by measuring the RI of the sample in a known solvent at various concentrations. The slope of the regression line of *n* versus *c* is then *dn/dc*.

For example, for a protein or complex that contains no carbohydrate, *dn/dc* is constant (often ≈ 0.185 mL/g) and nearly independent of the amino acid composition of the protein. However, it is advised to measure the actual *dn/dc* for each material in order to obtain an accurate result.

3.1.2 MEASUREMENTS AT MULTIPLE CONCENTRATIONS

When data are available at several concentrations, the second virial coefficient, A_2, may be determined (see *Equation 2*).

To determine the molecular weight, M_w, from measurements at different concentrations and at low angles [at which $P(\Theta) \approx 1$], use *Equation 2*:

$$\frac{K^* \times c}{R(\theta)} = \frac{1}{M_w} + 2A_2 \times c \quad (s)$$

By extrapolating the function $\frac{K^* \times c}{R(\theta)}$ to zero concentrations, the linear regression yields M_w as well as A_2:

$$Intercept = 1/M_w Slope = 2 \times A_2$$

However, the molecular weight obtained by this approach is very sensitive to the molecular polydispersity of the sample as well as interference by impurities.

3.1.3 COMBINATION WITH OTHER DETECTION TECHNIQUES

Determination of M_w by LALS stipulates that *dn/dc* is known. To this end, the RI increment, *dn/dc*, has to be measured for several different concentrations to generate a plot of *dn/dc* versus *c*, the *y*-intercept of which is *dn/dc*. Alternatively, when *dn/dc* is constant, the slope of the regression line of *n* versus *c* is *dn/dc*. Determination of *dn/dc* is facilitated by the coupling of LALS with an RI detector, and *Equation 4* is used for the RI signal, as shown below:

$$R_{RI}(\theta) = K_{RI} c M_w (dn/dc) \quad (4)$$

Combining *Equation 3* and *Equation 4* leads to *Equation 5*, as shown below:

$$M_w = K'(LS)/(RI) \quad (5)$$

where $K' = K_{RI}/[K_{LS}(dn/dc)]$ is the instrument calibration constant. The molecular weight M_w is then derived from the ratio of the *LS* versus the *RI* signal. This assumes that *dn/dc* is constant.

When *dn/dc* is no longer constant, for example in the case of carbohydrate-containing proteins, an additional detector is needed. For samples detectable by UV, a wavelength is chosen at which ε for the carbohydrate but not the protein part is = 0. Then the ε in *Equation 6* below is effectively that of the protein:

$$UV = K_{UV} C \varepsilon \quad (6)$$

The value of the specific refractive increment, *dn/dc*, can then be assumed to be proportional to *(RI)A/(UV)*. The outputs of the three detectors, designated *LS*, *RI*, and *UV*, respectively, are related to the molar mass of a sample according to *Equation 7*, as shown below:

$$M_w = \frac{K_{RI}^2}{K_{LS} K_{UV}} \times \frac{(LS)(UV)}{\varepsilon (RI)^2} \quad (7)$$

ε is a material but not an equipment constant and usually must be determined separately.

3.2 Multi-Angle Light Scattering

In MALS, measurements are made at multiple angles (at least three). To obtain the values of the three unknowns on the right-hand side of *Equation 2* (M_w, A_2, and the mean square radius, r_g^2), measurements of $R(\Theta)$ at three different angles are needed in order to obtain three independent expressions of *Equation 2*. The number of angles at which measurements are made (i.e., the quantity of data collected) determines the precision (reproducibility) as well as the accuracy of the measurement.

By measuring the angular dependence of the signal, it is possible to calculate the molecular size (r_g) from the initial slope (at small angles) of the angular dependence in *Equation 8*, as shown below:

$$\frac{d_y}{d_x} = \frac{16\pi^2 n_0^2 r_g^2}{3\lambda_0^2 M_w} \quad (8)$$

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where n_0 is the RI of the solvent, r_g is the root mean square radius (radius of gyration, molecular size), λ_0 is the vacuum wavelength of the laser, and M_w is the molecular weight.

In addition, measuring the signal over multiple angles provides information on the three-dimensional shape of the analyte, either by using non-linear regression analysis or graphically by using Zimm plots (*4*).

3.3 Combination of Light Scattering with Size-Fractionation Techniques

A more robust approach to molecular weight determination is based on pre-fractionation of poly-disperse protein mixtures or analytes into "slices" of uniform molecular weight. The coupling of fractionation and detection techniques affords more information than either method can provide on its own.

Size-fractionation techniques include size exclusion chromatography (SEC), field-flow fractionation (FFF) techniques, and other separation modes. By applying light scattering in combination with another concentration detector such as RI or UV, a more complete description of the molecular weight distribution can be realized.

Figure 2 shows an example of this for a sample of Dextran with a known molecular weight of approximately 40 kDa. The diagonal line shows the molecular weight across the SEC curve, and the entire distribution can be used to calculate molecular weight moments; the most obvious one is M_p, which is the molecular weight at the peak of the distribution. In addition, three other parameters can be calculated:

1. Number-average molar mass:

$$\overline{M_n} = \frac{\Sigma n_i M_i}{\Sigma n_i} = \frac{\Sigma c_i}{\Sigma c_i / M_i}$$

2. Weight-average molar mass:

$$\overline{M_w} = \frac{\Sigma n_i M_i^2}{\Sigma n_i M_i} = \frac{\Sigma c_i M_i}{\Sigma c_i}$$

3. *Z*-average molar mass:

$$\overline{M_Z} = \frac{\Sigma n_i M_i^3}{\Sigma n_i M_i^2} = \frac{\Sigma c_i M_i^2}{\Sigma c_i M_i}$$

The value of n_i is the number of molecules with molar mass M_i, and c_i is the weight concentration of molecules with molar mass M_i (as determined by the RI detector).

In the case of 40 kDa Dextran, the measured parameters are shown in *Table 1*, along with a calculation of the polydispersity ($I_p = M_w/M_n$).

Table 1. Measured Molar Mass Parameters for 40 kDa Dextran

Parameter	Value
M_n	29,420
M_p	34,650
M_w	40,760
M_z	55,960
I_p (= M_w/M_n)	1.385

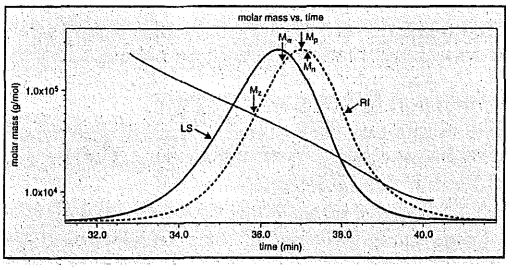

Figure 2. Molecular weight distribution for 40 kDa Dextran.

4. INSTRUMENTATION

Modern light scattering spectrometers use laser light because of the following advantages that lasers offer over conventional light sources:

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- Reliability
- Beam collimation
- Single wavelength (scattering depends on the wavelength of the scattered light)
- Equipment durability and compactness

Figure 3 shows a typical setup for the measurement of SLS. A monochromatic light source (i.e., a light source emitting light of only one wavelength; usually a laser) shines light on the sample. The intensity or the power of the scattered light is being measured at a known scattering angle Θ by a detector.

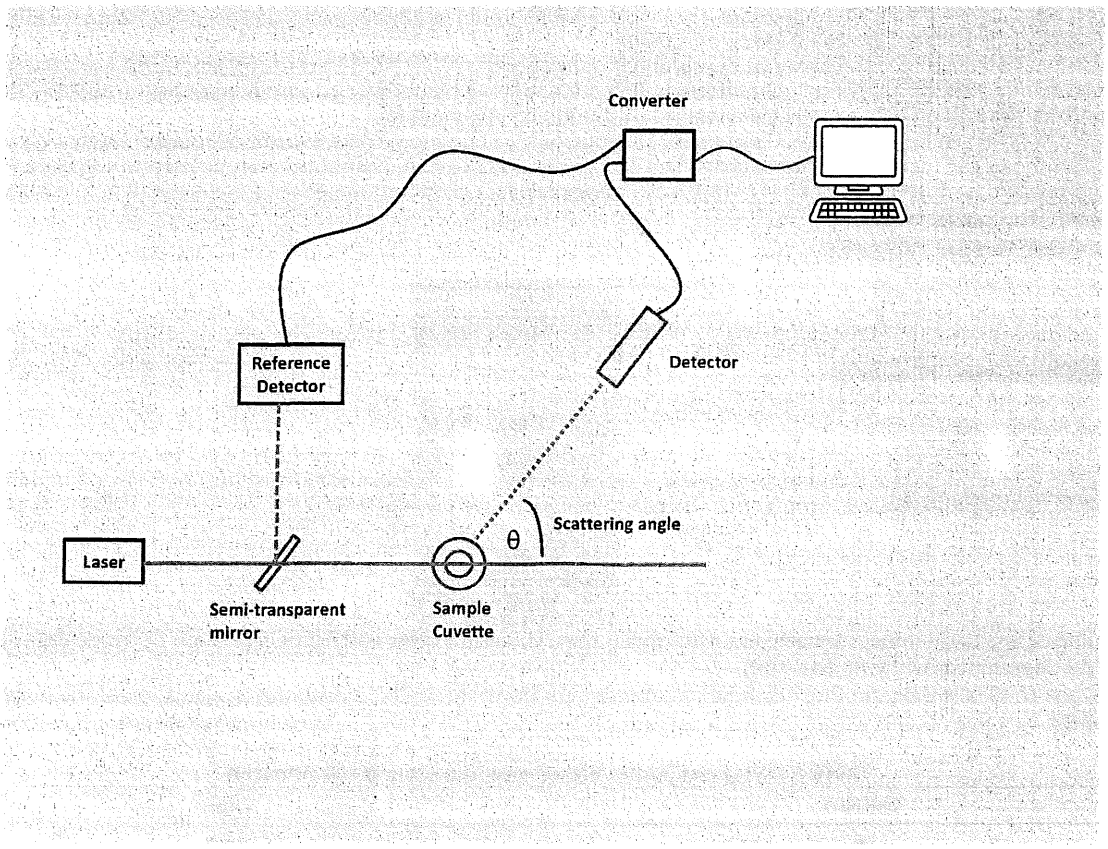

Figure 3. A typical setup of a static light-scattering instrument.

Historically, the measurements at different angles were made using a scanning photometer (single detector, variable angle). More recently, photometers containing a fixed array of 3–18 detectors have come into use.

5. LOW-ANGLE LIGHT SCATTERING VERSUS MULTI-ANGLE LIGHT SCATTERING

A major advantage of LALS is the exact measurement of high molecular weights (>10 million g/mol) without the principle-related errors that often occur with extrapolations, which are necessary for measurements at higher angles (but not with the use of many angles).

The major drawbacks of LALS when compared to MALS are as follows:

- **Higher noise:** Low angles are always noisier than high angles. Although using in-line filter(s) removes the scattering effects of debris from the low-angle data, it also removes much of the signal. Further, the measurement precision is roughly proportional to the square root of the number of detectors.
- **Less information:** Multi-angle data are needed to solve the Rayleigh equations.
- Inability to obtain information on molecular shape (see *3.2 Multi-Angle Light Scattering*).

LALS techniques and systems may be preferred for their simplicity and have been optimized to deal with their inherently lower information content (e.g., linearization strategies of multi-concentration data such as Zimm plots). Low-angle systems will be much cheaper and less dependent than the more complex equipment and (proprietary) fitting algorithms used in multi-angle systems. Finally, with the use of additional coupled simple detector systems such as RI and UV, the determination of molecular weight using LALS is a feasible, robust option.

A combination of the simplicity of LALS with the improved signal-to-noise ratio of higher scattering angles, offered by modern detectors, is achieved when performing measurements at Θ = 90°, the angle with the highest signal-to-noise ratio. Also, except

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for proteins of high molecular weight, there will be no significant angular dependence of the scattering with this configuration. However, for high molecular weight proteins, the light scattering signal has to be corrected with a viscosity detector, which requires making additional theoretical assumptions that are difficult to verify for routine applications.

6. PRACTICAL CONSIDERATIONS

Many SLS experiments, particularly those aimed at elucidating properties of proteins and other polymers in solution, will fall into the Rayleigh–Gans–Debye (RGD) scattering regime. *Table 2* summarizes the governing equations for four typical experimental situations: 1) low-angle scattering at a low concentration, which provides information on the molecular weight of the sample; 2) low-angle scattering at multiple concentrations, which provides information on molecular weight and solution non-ideality; 3) multi-angle scattering at a low concentration, which provides information on molecular weight and molecular size; and 4) multi-angle scattering at multiple concentrations, which provides information on molecular weight, molecular size, and solution non-ideality.

Table 2. Most Common Governing Equations

	Low Concentration	Multiple Concentrations
Low-angle light scattering (LALS)	$\frac{K^*c}{R(\theta)} = \frac{1}{M_w}$ Output: M_w only	$\frac{K^*c}{R(\theta)} = \frac{1}{M_w} + 2A_2c$ Output: M_w and A_2
Multi-angle light scattering (MALS)	$\frac{K^*c}{R(\theta)} = \frac{1}{M_w}\left(1 + \frac{q^2r_g^2}{3}\right)$ Output: M_w and r_g^2	$\frac{K^*c}{R(\theta)} = \frac{1}{M_w}\left(1 + \frac{q^2r_g^2}{3}\right) + 2A_2c$ Output: M_w, r_g^2, and A_2

Additional considerations:

- To obtain a reliable estimate for the excess Rayleigh ratio, one must use a calibration standard to obtain an "absolute" scattered intensity. Pure organic solvents (e.g., toluene) can be used for this purpose. It is important to note that the Rayleigh ratio of the solvent is a function of wavelength of the incident light. Alternatively, one can calibrate the system using a standard of known molecular weight (e.g., bovine serum albumin).
- For the above RGD equations to hold, samples must not absorb light in the wavelength range of the incident beam. Because laser light in the visible range is most common, samples should be colorless. If the samples are colored, more advanced data analysis techniques are required. Otherwise, the results will be unreliable.
- Likewise, cuvettes in the instrument systems with removable cuvette should not absorb light in the wavelength range of the incident beam. Care should be taken to ensure that the cuvette is free from smudges, scratches, or any other optical defect that could interfere with the measurement.
- Scattered intensity depends on the molecular size. Very small levels of large particles can dominate the signal and compromise the measurement. Samples should be appropriately prepared to minimize contamination from environmental or other sources. Sample preparation may include filtration, centrifugation, or other appropriate separation techniques. Care should be taken to ensure that the method of sample preparation itself does not introduce experimental artifacts (e.g., the analyte of interest adsorbing to a filter membrane). It is good practice to examine samples by dynamic light scattering to verify the absence of large contaminating species prior to conducting SLS measurements.

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Add the following:

▲⟨1430.2⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—LIGHT DIFFRACTION MEASUREMENTS OF PARTICLE SIZE

1. INTRODUCTION
2. THEORY
3. INSTRUMENTATION
4. APPLICATIONS
5. METHOD DEVELOPMENT
6. METHOD VALIDATION STRATEGY
7. DATA INTERPRETATION AND REPORTING

1. INTRODUCTION

Light scattering measurements may be used for the determination of particle size distributions because light scattered from particles has an angular dependence on the size of the particles. In principle, this application makes use of the same phenomenon as the low-angle light scattering (LALS) and multi-angle light scattering (MALS) techniques. The difference in the particle size measurement method lies in the higher spatial or angular resolution needed for the detection of scattered light. Light diffraction measurements allow for the derivation of a full-size distribution, with moderate to high resolution, rather than a single characteristic size. Specialized instrumentation and software have been developed for the specific needs of this application. Additional details on method and instrument requirements are provided in *Light Diffraction Measurement of Particle Size* ⟨429⟩.

Light scattered by particles in the near-forward direction is analogous to diffraction of light through an aperture. As this application makes use of the full pattern of diffracted light, it is properly described as light diffraction. It is also often referred to as laser diffraction due to the almost universal use of laser light sources.

The range of application, speed, minimal sample restrictions, and ease of use has made this the most broadly applied and commonly used technique for particle size analysis. Distributions with a resolution on the order of 100 independent channels are common. Measurements can be performed in seconds or less. Depending on the instrument configuration, the method is applicable to sizes ranging from about 0.01 µm to greater than 3000 µm. Particles may be measured in almost all matrices through which light can be transmitted, including liquids, gases, vacuums, and, potentially, solids.

The discussion in this chapter will focus on the application of laser light diffraction to materials of pharmaceutical interest. This still covers a wide range of materials, including excipients, active pharmaceutical ingredients (APIs), intermediates, starting materials, raw materials, in-process formulations, and drug products. Materials may be liquid, solid, inorganic, organic, crystalline, amorphous, single- or multi-phase, and combinations of these materials.

2. THEORY

The complete description of light scattering by spheres is given by the Mie theory, which is based on the solution to Maxwell's equation for the interaction of light with an optically homogeneous sphere. This accounts, not only for light diffracted by the particles, but also for light passing through the particles, i.e., refracted light. The Fraunhofer model represents a special case of the Mie theory that does not account for refraction. The assumptions here are that the refractive index (RI) of the particle material relative to the matrix and the absorptivity are high, so that effectively no light passes through. It also assumes that particles are large relative to the wavelength of the incident light. Thus, it is most properly applicable to particles greater than several micrometers for visible light sources. Though the Fraunhofer model can be applied when these conditions are not met, it can produce known artifacts and errors. However, it can still be useful as a default when the RI is unknown. The errors are often negligible for larger particles. A more detailed description of the Mie and Fraunhofer models can be found in ISO 13320:2009—*Particle size analysis—Laser diffraction methods*.

Some limitations in Mie theory are that it assumes (among other conditions) that particles are spheres with smooth surfaces and that they are optically homogeneous. These conditions are not generally met for most real particles of pharmaceutical materials. An additional complication is that most measurements are performed with the particles in motion. Thus, non-spherical particles may present a range of aspects to the incident light axis during the measurement, in effect appearing as multiple particles. Consequences of these deviations from theory are discussed in *7. Data Interpretation and Reporting*.

RI includes both real and complex components. The real component is commonly determined by either a microscopy method (Becke line technique) or from the RI of solutions of known concentration.

In the Becke line technique, particles are immersed in oils of known RI and observed with transmitted light. As the focus is moved above the plane of the particle, a bright ring may be observed moving into the region of higher RI, i.e., either into or

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away from the particle boundary. Using different oils, the particle RI may be determined well within the needed accuracy. Using polarized light, the RI of the individual crystalline axes may also be determined. In that case, an averaged value may be used in the Mie model.

In the solution method, an Abbe refractometer is used to measure the RI of a solution for which the concentration of the material is known. Extrapolating the solution RI, from a series of known concentrations of the material, to that of the pure substance yields a good approximation. This may require knowledge of the true density of the material.

The imaginary component of the RI is the absorptivity value for the wavelength of light used in the light diffraction measurement. For most non-colored materials, this will be very low. This value is not typically measured.

For both components, the values should be considered as initial approximations. Although the Mie model results are not especially dependent on the chosen values, it can sometimes yield unrealistic results. The sensitivity of the measurement to small changes in the assumed values should be assessed. This requires only the recalculation of an existing measurement using different RI values. Final RI and absorbance values should be chosen to avoid being too near an area of high sensitivity. The resulting size distributions should always be evaluated to assure that they realistically represent the sample.

3. INSTRUMENTATION

Instrumentation consists of light source(s), a measurement region, optics to collect the scattered and unscattered light, multi-element detector(s), and necessary hardware and software for the computations. Often more than one light source may be used. Light sources are usually lasers, though not always. Sources of different wavelengths are mainly used to extend the size range, especially to better handle samples in the submicron region.

The measurement region is where the sample is measured. Depending on the specific application, this is typically in a well-defined position relative to the detector and optics, and comprises windows that physically contain the sample dispersion. Associated sample dispersion equipment is also part of the instrument. (An exception would be for instruments specifically designed for sprays or on-line use.)

The detectors must provide adequate spatial resolution needed to best define the diffraction pattern. To best resolve the variations in diffraction patterns in the applicable size range, most elements are within a relatively low forward angle. Additional detectors at wider angles are often incorporated to extend the range to smaller sizes. Depending on the instrument design, different lenses may be used for specific size ranges. This is not common with the reverse Fourier configuration.

Further descriptions and illustrations of common instrumentation configurations are shown in ⟨429⟩ and ISO 13320.

4. APPLICATIONS

In principle, the light diffraction technique may be applied to any particulate system that allows for sufficient transmission of light: solid in liquid, liquid in liquid, gas in liquid, solid in gas, and liquid in gas. However, it is most commonly applied to solids dispersed in either liquids or gas (air), and to sprays of liquids or solids in air.

For solid powders, dispersion in either liquid or gas both offer advantages and disadvantages. Dispersion in liquids generally allows better control of the degree of dispersion of the material, which is important for understanding the desired characteristics of the powder. It also offers the advantage of being able to sample the dispersion's particles from the instrument dispersion outflow for observation by microscopy. Microscopy permits observation of the particles in the same state they exist during the light diffraction measurement. Obtaining particles from a dry dispersion as they exist in the dispersion would be difficult, if not impossible.

Disadvantages of liquid dispersion include possible changes in the material characteristics due to dissolution or flocculation. Selection of the dispersant system, including use of surfactants, is critical to developing a suitable method.

The advantages of dry or air dispersion of solid powders include the absence of solubility concerns and minimal sample preparation. A significant disadvantage is decreased control of particle dispersion. Though a range of dispersion energy can be applied by controlling the shearing air pressure, there is typically a reduced ability to separate deagglomeration from particle fracture processes. The occurrence of artifacts appearing as larger particles has also been observed, possibly due to material flaking from the surfaces of the dispersion unit. Larger sample sizes may also be needed.

Dry dispersion also requires adequate filtration and containment of the vacuum exhaust. Highly potent or toxic materials may not be suited for this analysis.

The use of dry dispersion is most appropriate for materials used in a similarly dispersed state, for example, the analysis of liquid or powder sprays from drug products and devices.

5. METHOD DEVELOPMENT

The fundamental step in starting the development of a method is to define the desired state of dispersion for the sample. Almost all powder samples will exhibit some degree of agglomeration. Attractions due to dispersion or van der Waals forces, electrostatics, or physical attachment, etc., are present in any sample. The strength of the attachments varies widely and depends on the particles' size, composition, and prior processing.

The terms agglomerate and aggregate will not be defined here. Within the scope of pharmaceutical materials, there can be an essentially continuous range of particle association strengths. Agglomerates or associations can range from those that are very weak and easily disrupted with minimal agitation, to those that are very strong and physically bound particles. Hence, the terms agglomerate and aggregate are sometimes used interchangeably, depending on the process used to create the material.

It is critical that the intended use of the particle size information be understood to determine the appropriate degree of dispersion. For example, measurement of primary, i.e., fully deagglomerated particles may be more informative for

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understanding the particle formation process or inherent dissolution behavior of a powder. Alternatively, including agglomerates in the measured distribution may be more useful for understanding issues of content uniformity and powder flow. A good understanding of the intended use of the material and expected forces encountered during further processing will help to determine the appropriate dispersion. Optical microscopy is an important tool to characterize the sample dispersion.

A typical dispersion study (assuming a solid in liquid dispersion) would first consist of analyzing a suspension created with minimal dispersion energy, e.g., by stirring or vortexing. Subsequent analyses of the suspension are performed after application of increasing dispersion energy, e.g., with added sonication times. If this is not possible with a single preparation, multiple preparations may be used. At each stage, the stability of the sample should be examined, e.g., with a stirring study. Comparing the resulting distributions with consideration of the sample behavior and supporting microscopy reveals the suitability of the dispersion conditions.

For dry dispersions, analyses using different shear air pressures and feed rates are done. This is often referred to as an air pressure titration study.

Once the intended state of dispersion is determined, method development will focus on how to best achieve and maintain that state reproducibly and reliably. For powders, dispersion either in air or a liquid is necessary. For liquids, the dispersant must support the dispersion of the particles to the desired extent, with no dissolution or other physical change or re-agglomeration. Though dissolution is not an issue for air or gas dispersion, achieving the proper degree of dispersion may be a greater issue.

The amount of incident light scattered or absorbed by the sample, expressed in terms of obscuration, transmittance, or optical concentration range of the sample, is related to the sample concentration and serves as a convenient proxy for relative concentration. It is expected that this will affect the measured size, though a range of values where the sensitivity of the results is low should be possible. This is generally a function of the overall instrument design and not material-specific. The lowest concentration must be sufficient to minimize the effects of noise, and the upper limit is needed to minimize the effects of multiple scattering. Suitable ranges may be recommended by the instrument manufacturer. Restricting the range may improve reproducibility.

6. METHOD VALIDATION STRATEGY

Chapter ⟨429⟩ provides guidance to demonstrate the required accuracy and precision for the instrument and methods of the laser light diffraction technique.

The International Council for Harmonisation (ICH) guidelines on method validation do not fully account for characterization methods such as particle size analysis. However, the fundamental concerns about method precision and robustness still apply.

As described in *Validation of Compendial Procedures* ⟨1225⟩, precision should be addressed by assessing repeatability and intermediate precision. Additional assessments of reproducibility could include multiple instruments and laboratory location.

The robustness assessment should address the sensitivity of the method to other likely sources of variation and should establish a range of acceptable conditions. Factors to consider will depend on the type of dispersion. For liquid dispersions, in addition to the classic stability considerations, this should include an assessment of the stability of the dispersion in the instrument over potential analysis times. Sample concentration, as indicated by obscuration, is an important factor, as it is most likely to have some effect. Other typical factors could include stability of the predispersion, recirculation or stir rate, dispersion energy, equilibration or stir time before initiating the measurement, and measurement time. For dry dispersion, measurement time, sample amount, air pressure, and feed rate to achieve target obscuration may be most important. These factors may be examined using an experimental design. For example, two or more factors could be evaluated by using high and low values at about the nominal value and determining the main effects.

Overall, the greatest sources of variations in the light diffraction technique are the sample properties, the sample preparation, and sampling. These far exceed any variations in the measurement process.

Given the fundamental aspects of the measurement process and the technique-dependent definition of size, the assessment of the accuracy is not simple. Comparison to an orthogonal technique is not always possible, especially given the different effects the particle shapes may have. A subjective assessment using optical or electron microscopy is recommended to compare the range of particle sizes observed with the reported light diffraction results.

7. DATA INTERPRETATION AND REPORTING

The ease of acquiring particle size distribution information by light diffraction belies potential pitfalls in its interpretation. Other than for perfect spheres, the concept of accuracy in describing the size of a single particle, much less a collection of varied particles, is elusive. The chosen optical model, particle shapes, and optical properties of the sample all influence the results of the measurement.

Calculations are based not on the sizing of any single particle but rather on the average diffraction pattern from the ensemble of particles passing through the measurement region over time. During this time, the particles can present multiple orientations.

The calculated size distribution is also not directly calculated from the diffraction pattern. Rather it is the result of comparing the cumulative theoretical patterns of individual size fractions, calculated using the chosen optical model, to the measured diffraction pattern. The relative proportions of the individual size are varied until the resulting calculated pattern best matches the measured one.

Thus the size distribution of a sample is best described as the size distribution of spheres with defined optical properties that produces a diffraction pattern equivalent to that of the sample. Interpreting the measured size distribution in terms of specific physical dimensions, e.g., length or width, is a mistake. Doing so has important implications for the interpretation of the reported size distribution, especially when considering the effects of particle shape on the results.

In some cases, the distribution of the modeled spheres may be restricted to a certain defined shape, e.g., a normal, log-normal, or Rosin–Rammler distribution. This was more common during the early stages of the instrument development,

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when computational abilities were more limited. This approach is not commonly used today, though the option may be available in the instrument software.

Because of how the results are derived, the particle size distribution from light diffraction measurement is best considered as the cumulative fraction of volume (or mass, assuming a uniform density) that is less than the defined sizes. This cumulative distribution is the basis for deriving the most commonly used statistics. These include percentile sizes such as ×50 and ×90 (the sizes for which 50% and 90%, respectively, of the sample volume are less than), as well as size quantiles such as Q10 (the fraction less than 10 µm). These are derived easily by interpolation from the cumulative distribution. For other statistics, such as various mean sizes, central values of the individual size bins must be assumed.

In plotting particle size distributions, if represented as a scatter plot, it should be realized that it is more properly considered as a histogram, with the relative height of each band representing the volume fraction within the size band, i.e., between the upper and lower limit. Unless the bins are evenly distributed on the size scale, the distribution density function versus size is what should actually be plotted. The distribution density is the volume fraction divided by the width of the size bin. This allows for the properly proportioned representation of a distribution when the size bands are of different widths. It will also permit measurements from different sources, with different numbers of bins to be overlaid on the same plot. One caution is that this quantity will change if the scale basis of the size axis is changed, e.g., from linear to logarithmic.

Further descriptions of particle size distribution statistics can be found in ISO 9276-1:1998—*Representation of results of particle size analysis—Part 1: Graphical representation* through ISO 9276-6:2008—*Representation of results of particle size analysis—Part 6: Descriptive and quantitative representation of particle shape and morphology.* ▲ (USP 1-Dec-2019)

Add the following:

▲⟨1430.4⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—ELECTROPHORETIC LIGHT SCATTERING (DETERMINATION OF ZETA POTENTIAL)

1. INTRODUCTION
2. THEORY
3. INSTRUMENTATION
4. APPLICATIONS
5. DATA ANALYSIS
    5.1 Calculation of Electrophoretic Mobility
    5.2 Calculation of Zeta Potential
6. DATA INTERPRETATION
7. ADDITIONAL SOURCES OF INFORMATION

1. INTRODUCTION

Zeta potential is a parameter that is primarily used to characterize the stability of suspensions and emulsions. It can also be used to study surface morphology and adsorption on particles and other surfaces in contact with a liquid. Zeta potential is indirectly determined by the appropriate theoretical models from experimentally determined parameters, such as electrophoretic mobility. Electrophoretic light scattering (ELS) is the most general and common way to determine zeta potential of colloidal systems.

2. THEORY

A suspension of particles that has a given electrokinetic charge is placed in a cell that has a pair of electrodes placed apart at a known distance. A potential of known strength is generated between the electrodes and, by electrophoresis, results in the particles migrating toward the electrode of opposite charge. To detect and quantify this migration, particles are illuminated by a laser and the Doppler shifts of the scattered light from particles are recorded. The difference between the frequencies of the incident light and the scattered light is used to determine the velocity and direction of the particle migration. The particle velocity is then used to calculate the electrophoretic mobility by taking into account the strength of the electric field. Then, taking into account the viscosity and permittivity of the base liquid, the electrophoretic mobility is used to calculate the zeta potential.

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3. INSTRUMENTATION

ELS, zeta potential instruments are light-scattering instruments that have either a reference beam optics alignment (most common) or a cross-beam optics alignment (less common). An example of a typical small-angle, light-scattering, zeta potential instrument (from ISO 13099-2:2012[1]) is shown in *Figure 1*.

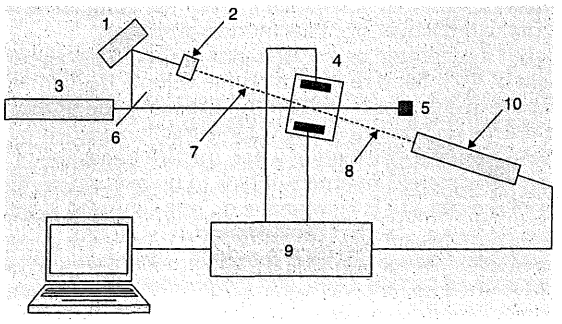

Figure 1. An example of a small-angle light scattering zeta potential instrument with reference beam optic arrangement.

Key	
1. Optical modulator	6. Beam splitter
2. Attenuator	7. Reference beam
3. Laser	8. Scattered or reference light
4. Sample cell with electrodes	9. Processor
5. Beam stop	10. Photoelectric detector

The cross-beam optical alignment instruments differ from the reference beam instruments in the fact that the laser beam is split into two beams of equal intensity that cross symmetrically (at a fixed angle between them) enter the sample cell from the same side.

A typical zeta potential measurement instrument is comprised of the following components:

1. A sample cell to contain the test suspension between two electrodes so that an electric field can be applied.
2. A suitable laser to illuminate the particles within the sample.
3. A suitable detector to capture the scattered light and the frequency shifts in the scattered light when the electric field is applied.
4. A software and computer system to process the collected scattering data and compute the mobility and zeta potential using appropriate models and algorithms.

The primary experimental technique used to determine zeta potential of colloidal systems is ELS. In this technique, a suspension is placed between two electrodes and an electric field is generated causing the ions and charged particles to migrate toward the oppositely charged electrode. When a laser is focused on the sample, this migration causes a Doppler shift in the frequency of the light scattered by the solution. An intensity or spectrum analysis of the shift in frequency is used to determine the electrophoretic mobility of the particles. Electroosmosis, the migration of the liquid along the sides of the cell flowing in the opposite direction from the particle migration, may affect the electrophoretic mobility measurement. A mechanism to correct for electroosmosis is often used during the measurement.

In cases where the Doppler shift is very small, the frequency analysis may not have enough resolution to determine the change. In such cases, phase analysis light scattering-based systems (PALS) can be used to perform the phase analysis of the shift. A typical schematic example of a PALS-based instrument is shown in *Figure 2*.

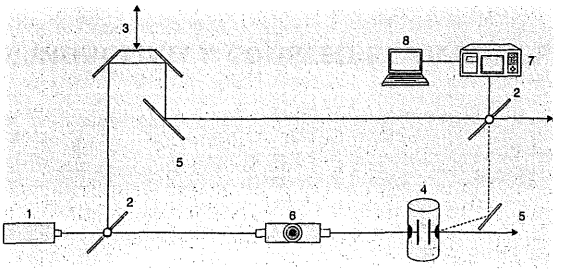

Figure 2. An example of PALS-based arrangement set-up of a zeta potential instrument.

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Key	
1. Laser	5. Mirror
2. Beam splitter	6. Variable attenuator
3. Pair of mirrors	7. Scattering detector
4. Sample cell with electrodes	8. Computer

The primary advantage of PALS is that it has higher resolution and can therefore detect very small Doppler shifts. This is typically needed for suspensions in nonpolar solvents or in aqueous solvents of high ionic strength in order to reduce Joule heating of the electrode surface. Elevated temperatures can cause changes in solution viscosity, which convolutes the measurement. Additionally, sample degradation can occur in some cases. The reported result from PALS is only the mean value of electrophoretic mobility. It does not provide any distribution information. Some current implementations combine PALS and spectrum analysis to obtain distribution information.

4. APPLICATIONS

In general, ELS is used to characterize the charge distribution (or average charge) and stability of suspensions and emulsions. ELS has been used widely for characterizing colloidal systems where it is important to control their stability (e.g., flocculation of submicron materials suspended in a liquid). Examples include preparations of pharmaceutical suspensions and emulsions where flocculation could affect appearance, efficacy, and bioavailability. In biological applications and in the biotechnology industry, ELS was used early on to characterize the surface properties of cells. More recently, the biopharmaceutical industry has adopted ELS measurements during development of formulations for the evaluation of degradation pathways and the characterization of compositional variants.

5. DATA ANALYSIS

5.1 Calculation of Electrophoretic Mobility

The ELS method is used to determine the electrophoretic mobility (μ) by measurement of the Doppler frequency shift (Δω) of the scattered light in an applied electric field (*E*) using either *Equation 1* (for the reference beam optical alignment) or *Equation 2* (for the cross-beam optical alignment):

$$\mu = \frac{\Delta\omega\lambda}{4\pi n E \sin\left(\frac{\theta}{2}\right)\sin\left[\left(\frac{\theta}{2}\right)+\xi\right]} \qquad (1)$$

$$\mu = \frac{\Delta\omega\lambda}{4\pi n E \sin\left(\frac{\theta'}{2}\right)} \qquad (2)$$

Δω = Doppler frequency shift
λ = laser wavelength in vacuum
n = refractive index of the medium (or base liquid)
E = electric field strength
θ = angle between the incident light and the scattered light
ξ = angle between the scattered light and the orientation of the electric field
θ′ = angle between the two beams

5.2 Calculation of Zeta Potential

For non-conductive spheres, zeta potential (ζ) is commonly calculated using an extension of the Henry equation for its relationship with electrophoretic mobility (μ) (*Equation 3*).

$$\mu = \frac{2\zeta\varepsilon}{3\eta_0} f(\kappa\alpha) \qquad (3)$$

ε = dielectric permittivity of the medium (or base liquid)
η_0 = viscosity of the medium (or base liquid)
κ = reciprocal of the Debye double-layer thickness
α = sphere radius
$f(\kappa\alpha)$ = a monotonic function varying from $f(\kappa\alpha) = 1$, as $\kappa\alpha \to 0$, to $f(\kappa\alpha) = 3/2$, as $\kappa\alpha \to \infty$

When $\kappa\alpha >> 1$, typical for large particles in aqueous suspensions, $f(\kappa\alpha) = 3/2$ in the above formula, leading to the Smoluchowski equation (*Equation 4*).

When $\kappa\alpha << 1$, typical for small particles in organic liquids, $f(\kappa\alpha) = 1$ in the above formula, leading to the Hückel equation (*Equation 5*).

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$$\zeta = \frac{\eta_0 \mu}{\varepsilon_r \varepsilon_0} \quad (4)$$

$$\zeta = \frac{3\eta_0 \mu}{2\varepsilon_r \varepsilon_0} \quad (5)$$

Thus, depending on the nature of the particles and suspending medium, zeta potential (ζ) is calculated from the electrophoretic mobility (μ) utilizing either the Smoluchowski equation (*Equation 4*) or the Hückel equation (*Equation 5*).

The Smoluchowski equation is used when large solute particles (the hydrodynamic radius is large compared to the thickness of the electrical double layer) are probed, whereas the Hückel equation is preferred when small solute particles (the hydrodynamic radius is small compared to the thickness of the electrical double layer) are probed.

6. DATA INTERPRETATION

Zeta potential, expressed in units of millivolts (mV), is primarily used to characterize the physical stability of a suspension/emulsion. An unstable suspension may result in flocculation and sedimentation, and an unstable emulsion may collapse and result in phase separation. In general, as the absolute value of zeta potential decreases in magnitude, the suspension may become less stable and vice versa. The stability evaluation must be performed in conjunction with other relevant techniques. Although the relative stability change of the suspension may be indicated by changes in the value of the zeta potential, there is no direct link between zeta potential and absolute stability. Moreover, multiple determinations of different lots or preparations may be necessary to make general conclusions regarding the stability of different suspensions.

Although zeta potential is commonly used to quantitatively characterize the stability of a suspension, it is not intended to be the sole indicator of stability. Typically, zeta potential data are coupled with other quantitative or qualitative characterization techniques to monitor and characterize suspension stability, such as monitoring appearance or turbidity over time.

Zeta potential is also relative to the composition of the suspension. Typically, there is no distinct value of zeta potential that can be used as a threshold of stability.

It is important to note that zeta potential is a characteristic of a suspension, and not just of the particles. This includes the particles and the surrounding liquid environment with any ions or chemicals present in equilibrium. If care is not taken, performing multiple dilutions may alter this environment and change the zeta potential. It is also important to note that since the measurement relies on the migration of particles in an electric field, performing repeat measurements (NMT 3–5 depending on the sample) on the same aliquot may cause a shift in the zeta potential. If significant change is observed, an orthogonal method may be used to verify the integrity of the sample. Also note that a change of the zeta potential of a suspension may also change the observed hydrodynamic particle diameter by dynamic light scattering.

7. ADDITIONAL SOURCES OF INFORMATION

- International Organization for Standardization. ISO 13099-2:2012: Colloidal systems—Methods for zeta potential determination—Part 2: Optical methods.
- Xu R. Electrophoretic light scattering—zeta potential measurement. In: Xu R, ed. *Particle Characterization: Light Scattering Methods*. New York, NY: Kluwer Academic Publishers; 2002:289–343. ▲ (USP 1-Dec-2019)

Add the following:

▲⟨1430.5⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—SMALL ANGLE X-RAY SCATTERING AND SMALL ANGLE NEUTRON SCATTERING

1. INTRODUCTION
    1.1. Applications
2. TYPES OF SAS
3. BASIC THEORY
4. CONTRAST MATCHING
5. INSTRUMENTATION
6. EXPERIMENTAL CONSIDERATIONS
    6.1 Incident Flux
    6.2 Resolution
    6.3 Instrument Calibration
    6.4 Sample Quality
    6.5 Sample Concentration
    6.6 Sample Containers

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6.7 Backgrounds
6.8 Counting Times
6.9 Sample Transmission
6.10 Multiple Scattering
6.11 Radiation Damage
6.12 Sample Deuteration
7. DATA PROCESSING
7.1 Data Reduction
7.2 Data Analysis
8. ADDITIONAL SOURCES OF INFORMATION
REFERENCES

1. INTRODUCTION

Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) make up a sub-family of small-angle scattering (SAS) diffraction techniques suitable for probing the size, shape, ordering, and interactions of molecules and molecular assemblies on length scales that are typically from around 1 nanometer (nm) to a few hundred nm. This size range overlaps with the lower range probed using the laser light diffraction technique (see *Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size* ⟨1430.2⟩).

Because these length scales are much larger than inter-atomic distances, in crystallographic terms SAS is considered low-resolution diffraction. Nonetheless, it is an extremely useful and versatile technique capable of providing structural information complementary to cryogenic transmission electron microscopy (cryo-TEM), high-resolution nuclear magnetic resonance (NMR), and analytical ultracentrifugation.

SAS differs from dynamic light scattering (DLS; see *Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering* ⟨1430.3⟩) in that it is a direct probe of size and shape.

1.1 Applications

SAS is routinely performed on solids, liquids, and dispersion samples (both aqueous and nonaqueous). Some types of SAS have been used to study foams.

In terms of pharmaceutical applications, SAS has been used to study topical creams, intravenous emulsions, hydrogels, polymer-drug conjugates, proteins, enzymes, and antibodies (*1–5*). Some types of SAS are particularly well suited to studying the physical chemistry underlying formulation problems.

SAS can be used in various applications to evaluate the size, shape, and ordering of molecules. For example, SAS can provide information on the size and dispersity of droplets, macromolecules, micelles, liposomes, nanoparticles, crystalline domains, and pores (including in mixtures of these and other multi-phase systems). With regard to shape, SAS can be used to determine the shape of micelles and nanoparticles; the tertiary structure of proteins, enzymes, and antibodies (using SAS-constrained modeling); and the conformational distribution of adsorbed layers. Finally, examples of the use of SAS to assess molecular order include determining the core-shell structure of micelles and sterically stabilized nanoparticles, the bilayer structure in liposomes, and the liquid crystalline structure (e.g., lattice parameters, space groups).

2. TYPES OF SAS

SAS is a specialized, but nonetheless highly useful and versatile, technique. Although a detailed discussion of SAS is beyond the scope of this chapter, the intention of this chapter is to raise awareness and then direct the reader to authoritative sources for further information.

SAS is most commonly performed using light [small-angle light scattering (SALS)], X-rays (SAXS), or neutrons (SANS). It is the wavelength of these different radiation sources, and the way that the radiation interacts with the atoms in a sample, that determines what type of information is obtained in a particular SAS experiment. Importantly, data from all of these variants of SAS can be analyzed using common formulae and software packages. *Table 1* summarizes some of the key aspects of the different types of SAS.

Table 1. Key Aspects of Different Types of Small-Angle Scattering

	SALS	Lab-SAXS	Synchrotron-SAXS[a]	SANS[a]
Radiation scattered by	Electrons	Electrons	Electrons	Nuclei
Radiation velocity (m/s)	3×10^8	3×10^8	3×10^8	10^2–10^3
Accessibility	Laboratory	Laboratory	Synchrotron source	Neutron source
Relative brilliance	Medium	Medium	High	Low
Wavelengths (nm)	400–700	0.07–0.23	0.06–0.33	0.15–2.5
Length scales probed (nm)	250–25,000	≤1–350	0.1–2500	0.2–1000+

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Table 1. Key Aspects of Different Types of Small-Angle Scattering *(continued)*

	SALS	Lab-SAXS	Synchrotron-SAXS[a]	SANS[a]
Impact of dust contamination	Significant	Negligible	Negligible	Negligible
Effect of hydrogen (H)/deuterium (D) substitution	None	None	None	Large
Radiation/heat damage	Negligible	Possible	Very likely	Negligible
Absolute intensity calibration	Possible	Possible	Possible	Routine
Sample volumes (mL)	0.05–5	0.007–0.1	0.0001–0.5	0.1–5.5
Study optically opaque samples	No	Yes	Yes	Yes
Typical container materials	Glass, quartz	Polyimide (Kapton), polycarbonate, beryllium, silica/quartz, silicon nitride	Polyimide (Kapton), polycarbonate, beryllium, silica/quartz, silicon nitride	Silicon, silica/quartz, aluminum, titanium, vanadium, copper silicon nitride

[a] Access to national and international large facilities is usually obtained through competitive peer review, but experimental time for successful proposals is then free, provided that the results are placed in the public domain. Where this route of access is unsuitable, for example, for commercial customers who require confidentiality or a guarantee of experimental time, a range of paid industrial access mechanisms will typically be available.

3. BASIC THEORY

When a beam of radiation is directed at a sample, a fraction of the beam will be transmitted, a fraction will be absorbed (and in some experiments may then be re-radiated), and the remainder will be diffracted through a range of angles, Θ. In a typical SAS experiment, $0.01° < \Theta < 10°$; this value of Θ is different from that in wide-angle and classical crystallographic diffraction experiments, where $\Theta > 10°$. On some instruments, it may be possible to record the small- and wide-angle data concurrently.

Radiation scattered through the same angle but originating from different points in the sample may then superimpose constructively or destructively at the detector to construct a "scattering pattern". This reflects spatial fluctuations in the refractive index (RI) (SALS), electron density (SAXS), or scattering length density (SANS) of the sample, which in turn arise from differences in the size, shape, and order of the constituent molecules.

The length scale, d, of the spatial fluctuations that may be accessed in a SAS experiment is determined by Bragg's Law:

$$d = \frac{\lambda}{2\sin(\theta)}$$

where λ is the wavelength. Experimentally, it is more typical to express this in terms of (the modulus of) a quantity called the "scattering vector", q:

$$q = \frac{4\pi n}{\lambda}\sin(\theta) = |q| = |k_s - k_i|$$

from which it follows that:

$$d = \frac{2\pi n}{q}$$

where k_i and k_s are the incident and scattered wave vectors and n is the RI of the medium. Conveniently, for neutrons and for X-rays, n may be assumed to be unity. Note that q is an inverse length scale. The geometry of a SAS experiment is illustrated in *Figure 1*.

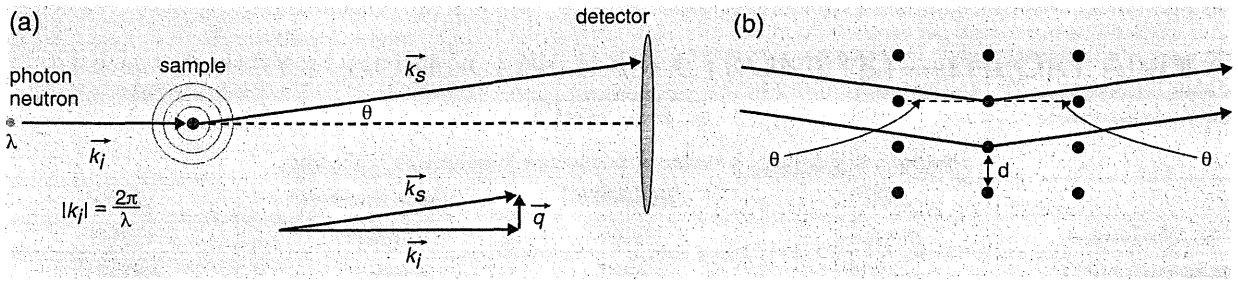

Figure 1. (a) The geometry of an SAS experiment, where k_i and k_s are the incident and scattered wave vectors and q is the resultant scattering vector. (b) Schematic representation of diffraction from atoms in a sample.

The number of photons/neutrons of a given wavelength, scattered through a particular angle, that arrive at a detector pixel in a unit time, $N(\lambda,q)$, may be expressed as:

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$$N(\lambda,q) = N_0(\lambda)\Delta\Omega\,\eta(\lambda)T_r(\lambda)V_S I(q)$$

where N_0 is the incident flux of photons/neutrons, $\Delta\Omega$ is the solid angle element defined by the size and distance of the detector pixel from the sample, η is the detector efficiency, T_r is the transmission of the sample (i.e., 1 – absorbance), V_S is the volume of the sample illuminated by the beam, and $I(q)$ is the scattering function. Note that the first three terms are instrument-specific, whereas the last three terms are sample-dependent. The scattering function, the quantity determined in an SAS experiment, is given by:

$$I(q) = \Phi V(\Delta\rho)^2 P(q) S(q) + B$$

where Φ is the volume fraction of the scattering objects in the sample (e.g., the volume fraction of nanoparticles in a dispersion); V is the volume of one scattering object; $\Delta\rho = (\rho_{object} - \rho_{matrix})$ is the contrast of the sample (discussed below); the form factor $P(q)$ describes how the scattering is modulated by the size, shape, and dispersity of the scattering objects; the structure factor $S(q)$ describes how the scattering is modulated by interactions; and B is a residual background signal normally taken to be q-independent. $P(q)$ has a value between 0 and 1. As $\Phi \to 0$, so $S(q) \to 1$.

For a solute in solution:

$$\Phi V = \frac{cM}{N_A \delta^2}$$

where c is the mass concentration of the solute, M is its formula weight, and δ is its bulk density. The objective of an SAS experiment is to extract information from $P(q)$ and/or $S(q)$. How this is achieved is discussed later. However, every component in the sample, including the container, will contribute to the overall scattering from the sample. So in addition to obvious background correction measurements (e.g., empty container; container + solvent), it is often useful to manipulate the various other individual contributions.

4. CONTRAST MATCHING

From the discussion in the preceding section, it is apparent that if the contrast term is zero, there will be no SAS. This condition is known as "contrast matching" and is potentially extremely useful because it permits the simplification of the scattering from complex multi-component samples and the study of individual components.

In SALS, contrast matching requires that two or more components have the same RI. The reader may have experienced this phenomenon when washing a glass in a bowl of water; the glass can seem to disappear because the glass and the water have similar RIs. In many experiments, however, RI matching of the components in a sample is often difficult to achieve in a useful manner. Some SALS and DLS instruments, however, use index-matching baths around the sample cell to reduce scattering from the outer wall of the cell.

In SAXS, contrast matching requires that two or more components have the same electron density. Because the electron density of a molecule is strongly influenced by the atomic number of the elements it contains, contrast matching a molecule with a heavy atom, such as a cationic surfactant, to an aqueous medium is not possible unless an additional solute is added to bolster the electron density of the medium. However, this course of action may change the pristine characteristics of the analyte and its chemical environment.

Contrast matching is more useful in SANS. Here, contrast matching is achieved if two or more components have the same scattering length density, ρ:

$$\rho = \frac{\delta N_A}{M}\sum_i b_i$$

where b_i is the (bound) "coherent neutron scattering length" of nucleus i, and the other variables are as defined previously. The summation only needs to be performed over the empirical formula for a component.

Scattering length density calculators can be found online or as tools in some SAS analysis programs.

A compendium of neutron scattering lengths is available online (*6*).

An example of a calculation for poly(ethylene oxide), $–[CH_2\text{-}CH_2\text{-}O]_n–$:

C_2H_4O, M = 44 g/mol, δ = 1.127 g/cm³, b in cm

$$\rho = \frac{1.127 \times 6.023 \times 10^{23}}{44} \times \{(2 \times 6.646 \times 10^{-13}) + (4 \times -3.739 \times 10^{-13}) + 5.803 \times 10^{-13}\}$$

$$\rho = 0.64 \times 10^{10}\,\text{cm}^{-2} \text{ or } 0.64 \times 10^{-6}\,\text{Å}^{-2}$$

Importantly, because they depend on the neutron–nucleus interaction, scattering lengths are isotope-dependent. The same calculation for perdeuterated poly(ethylene oxide), for example, would yield $\rho = 6.46 \times 10^{-10}$ cm^{-2}.

[NOTE—It is a coincidence that this value appears to be 10 times the hydrogenous value.]

Thus, D–for–H exchange offers significant opportunities for exploiting contrast matching in soft-matter SANS experiments.

Table 2 lists some illustrative scattering length densities. These may sometimes be found expressed in the equivalent but non-SI units of 10^{-6} Å^{-2}.

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Table 2. Neutron Scattering Length Densities of Pharmaceutically Relevant Molecules

Molecule	$\rho(10^{10}cm^{-2})$	Molecule	$\rho(10^{10}cm^{-2})$	Molecule	$\rho(10^{10}cm^{-2})$
H_2O	−0.56	H_{25}–sodium dodecyl sulfate (SDS)	0.33	Lipids	0.1–0.4
D_2O	6.37	D_{25}–SDS	5.83	Proteins	2.2–2.6
H_8–toluene	0.94	H_{60}–Triton ×100	0.57	Carbohydrates	2.7
D_8–toluene	5.66	H_{31}–dodecyldimethylamine oxide (DDAO)	−0.20	DNA	4.0
Perfluorodecalin	4.21	H_{39}–deoxycholate Na	0.66	RNA	4.5

Several useful characteristics of scattering length densities are evident in the table. First, they can be positive, negative, or, in principle, zero. Second, different classes of biological molecules span a wide range of scattering length densities. Lastly, it is usually possible to find a solvent, or preferably a mixture of solvents, that will contrast match any solute. So, for example, DNA can be contrast matched in a mixture of 34.1% H_2O: 65.9% D_2O by volume, and a perfluorodecalin-in-water emulsion would not scatter neutrons if the oil was dispersed in a mixture of 31.1% H_2O: 68.9% D_2O by volume. It is even possible to make an H_2O:D_2O mixture that does not scatter (i.e., $\Delta\rho = 0$). *Figure 2* shows how contrast matching could be used in a SANS study.

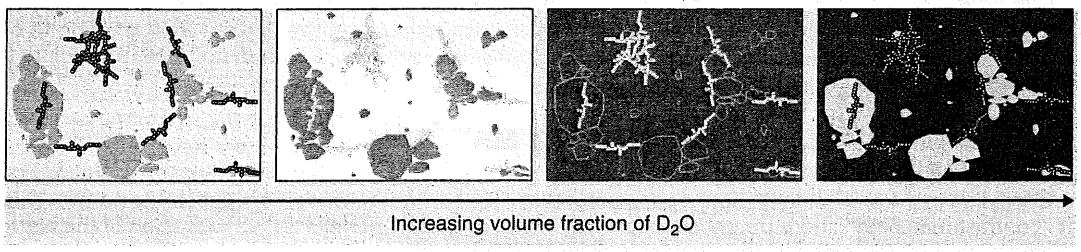

Figure 2. Schematic illustration of how contrast matching might be used in a hypothetical SANS study of a dispersion containing polymer/surfactant and drug particles. The scattering from all four figures would be different. The first and last would differ only in overall intensity, but the form of the scattering from these would differ from the forms of the second and third figures, which highlight different components of the sample.

When calculating scattering length densities, it is important to use as accurate a value as possible for the bulk density, δ. However, sometimes determining δ can be problematic for deuterated compounds. In such instances, a useful rule of thumb for solvents and relatively simple molecules is $\delta_D = \delta_H \times 1.1$. Also, it is important to remember that δ is temperature-dependent.

Molecules containing hydroxyl groups, carboxylic acid groups, and even some amine groups will readily exchange those functional protons for deuterons when exposed to D_2O. This means that the scattering length density of some molecules can be pH-dependent; proteins are an obvious example. Some scattering length density calculators include a degree of exchange parameter to compensate for this pH dependence.

5. INSTRUMENTATION

The components of a SAS system (see *Figure 3*) are common across the different forms of the technique.

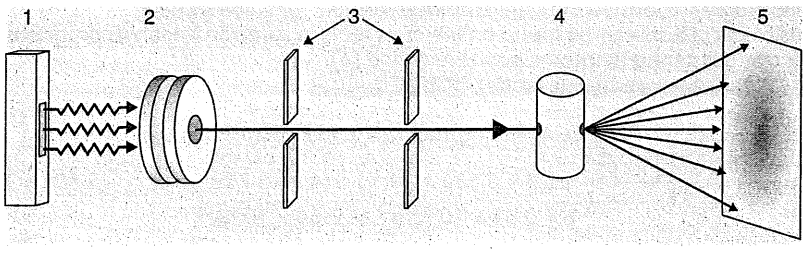

Figure 3. Schematic representation of a generic SAS instrument.

Key	
1. Source 2. Optics 3. Slits	4. Sample 5. Detector

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SALS and SAXS are performed using monochromatic radiation, which is collimated with slits or pinholes to the desired profile regarding size (diameter or width/height) and shape (line or point). The wavelength of monochromatic radiation is determined by the choice of source (replacing one type of anode material with another), but may also be varied by changing optical filters, or adjusting the synchrotron monochromator. SANS at reactor neutron sources is also performed in this manner, with the wavelength adjustment performed by a rotating device called a velocity selector. In these fixed-wavelength measurements, altering the dynamic range in q of the instrument requires changing the sample-to-detector distance (either along the incident beam direction or off-axis) in order to change the range of scattering angles subtended to the sample.

However, SANS can also be performed at pulsed neutron sources (based on particle accelerators—so-called spallation sources—or pulsed reactors). At these sources, a "polychromatic" ("white") beam of neutrons is directed at the sample, and the time of flight (TOF) of the scattered neutrons to the detector is recorded. This establishes the neutron velocity and thereby its wavelength. The dynamic range in q of these TOF-SANS instruments is much broader than that of their fixed-wavelength counterparts because every wavelength that reaches a pixel on the detector corresponds to a different q value, although a given pixel on the detector still represents a single scattering angle. Moreover, the intrinsic wavelength resolution, and therefore q-resolution, of TOF-SANS is much better than that of fixed-wavelength SANS.

The scattering from a sample actually occurs over 4πspace, but because an SAS instrument is linear, the detector only intercepts a conic section of the total scattering. Because most soft matter samples have some degree of intrinsic disorder (referred to as "polycrystallinity" by crystallographers), the detector records multiple rings of varying intensity rather than discrete spots (reflections) (see *Figure 4*).

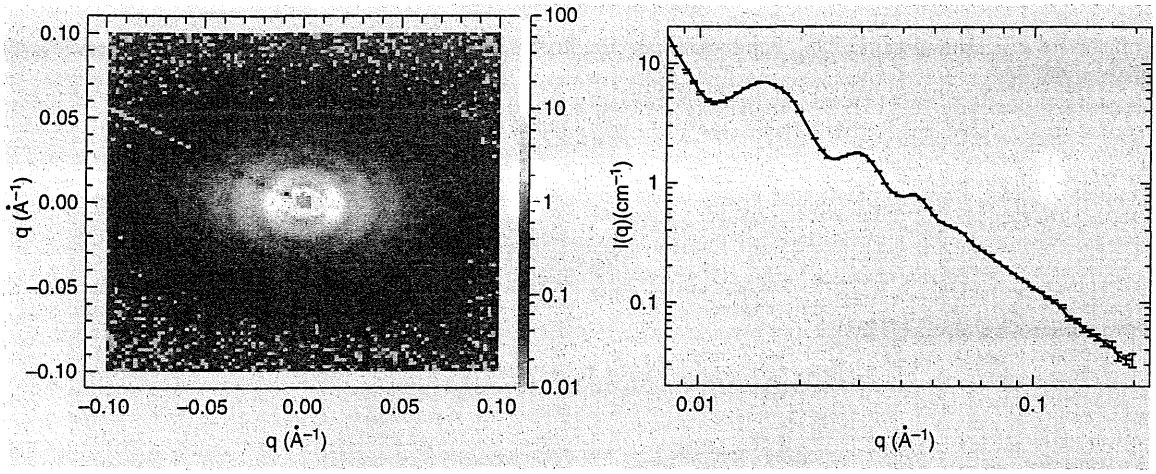

Figure 4. Two representations of the SAS (in this case, SANS) from the same aqueous dispersion of elongated surfactant micelles. The sample has gelled to form a lyotropic liquid crystalline phase exhibiting lamellar order (at least 4 orders of diffraction are visible as rings or peaks). The lamellae have then aligned vertically (giving rise to an anisotropic 2D pattern along the equatorial direction on the detector) as a result of the shear field imposed when the sample was injected into the sample cuvette.

The scattered radiation will usually be detected by solid-state charge-coupled device (CCD) cameras (SALS/SAXS), gas detectors (SAXS/SANS), or scintillator detectors (SANS). Often these will be highly pixelated two-dimensional ("area") detectors, which are extremely useful for resolving anisotropic scattering from oriented samples. A state-of-the-art SAXS detector for laboratory applications may have 1 thousand (for one-dimensional detectors) to more than 1 million pixels (for two-dimensional detectors), and more for large-facility instruments. Consequently, the data volumes generated during an experiment may be quite large (gigabytes to terabytes).

In order to allow them to access smaller angles, and because they require more substantive radiation shielding, SAXS/SANS instruments at large facilities may extend several tens of meters from the source. Even a state-of-the-art laboratory SAXS instrument is typically 3–5 m in length. While in principle one could attain ever-smaller q values by building very long instruments, in practice the count rate at the detector declines as the inverse-square of the sample–detector distance; thus, measurements become impractically slow. To circumvent this limitation and permit very-small-angle scattering (VSAXS/VSANS) or ultra-small-angle scattering (USAXS/USANS) measurements, alternative strategies are employed, such as using focusing optics or slit-collimated Bonse–Hart "double–crystal" geometries. These instruments can access length scales (d) into the micron scale, i.e., the same realm accessible by optical microscopy. The type of source collimation, point-source (pinhole) versus slit, has implications for data processing. Very recently, a new form of SANS has emerged wherein the scattering angle is encoded in the precession of polarized neutrons. This technique, spin-echo (modulated) SANS (SESANS/SEMSANS), can also access micron-length scales but measures in d-space rather than in q-space. Its advantages over Bonse–Hart VSANS are that it retains good count rates and is much less susceptible to multiple scattering from the sample. Further discussion of these extended SAS techniques is beyond the scope of this chapter, but the reader should be aware of their existence.

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6. EXPERIMENTAL CONSIDERATIONS

Performing a successful SAS experiment depends on many factors, including but not limited to the following factors described in subsections *6.1–6.12* (*7–9*).

6.1. Incident Flux

Incident flux is the number of photons/neutrons per unit area per second delivered to the sample. It is a function of the brilliance of the source (see *Table 1*) and the wavelength selection/collimation employed for the experiment. Kinetic or dynamic measurements, or measurements on very weakly scattering samples, will fare better on higher-flux instruments.

6.2 Resolution

There are two types of resolution of relevance to an SAS experiment:

1. Size resolution of the instrument
2. q-resolution of the measurement

6.2.1 SIZE RESOLUTION

The maximum, d_{max}, and minimum, d_{min}, length scales of the structure to be probed need to lie inside the measurement limits of the instrument, i.e.:

$$q_{min} < \frac{\pi}{d_{max}}$$

and

$$q_{max} > \frac{\pi}{d_{min}}$$

This principle is illustrated in *Figure 5*.

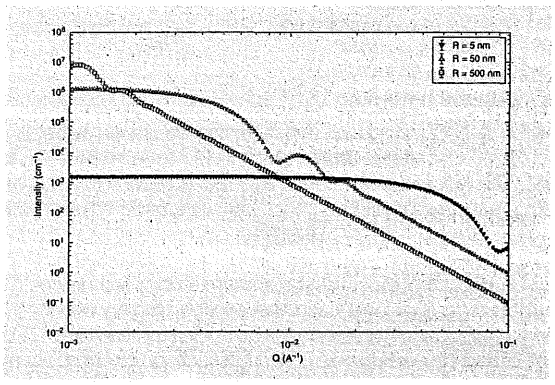

Figure 5. Calculated scattering for three dispersions of perfectly spherical homogeneous particles of the same material in the same matrix, differing only in the particle radius, R. A 10% log-normal particle size distribution has been applied to aid realism. The curves are displaced vertically to aid clarity. Only the scattering from the $R = 50$ nm particles is fully within the measurement window of this simulated instrument, as shown by the limiting behavior of the form factor at low Q and multiple interference fringes at intermediate Q. Also note the q-4 dependence of the high-q scattering, a manifestation of Porod's Law arising from the particle surfaces. Down triangles, $R = 5$ nm; up triangles, $R = 50$ nm; squares, $R = 500$ nm.

6.2.2 q-RESOLUTION

The q-resolution is the precision with which adjacent q-points can be distinguished, and therefore it governs the level of fine detail in the measured scattering data that can be resolved (for example, the peaks in *Figure 4*).

The q-resolution is primarily determined by the wavelength resolution of the instrument, then by the geometric resolution arising from the selected instrument collimation, and then by a range of other less significant factors.

Laser light sources and X-ray sources have the best wavelength resolution, with $\Delta\lambda/\lambda$ values as low as 0.01%. Conventional light sources depend on the use of a bandpass filter for which $\Delta\lambda/\lambda$ may be ~1%. Although good wavelength resolution is an intrinsic property of a laser, in the case of X-rays, it is determined by the monochromator in use. The wavelength resolution of fixed-wavelength SANS instruments depends on the specification of the neutron velocity selector employed. Typically, this resolution will be between 8% and 20%. However, in TOF-SANS the wavelength resolution is determined by the precision with

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which the neutron TOF (typically a few thousand microseconds) can be measured, and thus $\Delta\lambda/\lambda$ values of approximately 5% would not be uncommon. In most instances, better wavelength resolution can always be traded for reduced flux and vice versa.

The geometric resolution is essentially governed by the beam size at the sample; a smaller size is better. Laser-based SALS and SAXS instruments with submillimeter beam footprints fare much better than SANS instruments in this regard, where a relatively small beam may still be 6–8 mm in diameter. This difference is further compounded by the fact that because neutrons cannot be easily focused, reducing the beam footprint also decreases the flux on the sample.

6.3 Instrument Calibration

SAS instruments require two separate calibrations, but with an astute choice of calibration sample it might be possible to perform both calibrations in one measurement:

1. Calibration of the q-scale
2. Calibration of the intensity scale

An instrument technician or, at large facilities, the assigned experiment local contact, will normally perform these calibrations. However, if one's plan is to use several different SAS instruments it is advisable to acquire and measure one's own calibrants to ensure confidence in the local calibrations and ensure that all of one's data are on self-consistent scales.

6.3.1 q-CALIBRATION

It is possible to omit the q-calibration on a fixed-geometry SAS instrument where the sample-to-detector distance is constant/known. In all other instances, it is necessary to measure a sample with a characteristic q–dependence related to structure that can be unambiguously verified by other means [e.g., TEM, atomic force microscopy (AFM), etc.]. Alternatively, if an SAXS instrument has an energy-responsive detector, and an accurate determination of scattering angle is possible, then an absorption edge can be used to verify q. Two commonly used q-calibrants for SAXS/SANS are silver behenate (AgBH) (see *Figure 6*) and rat tail collagen (RTC).

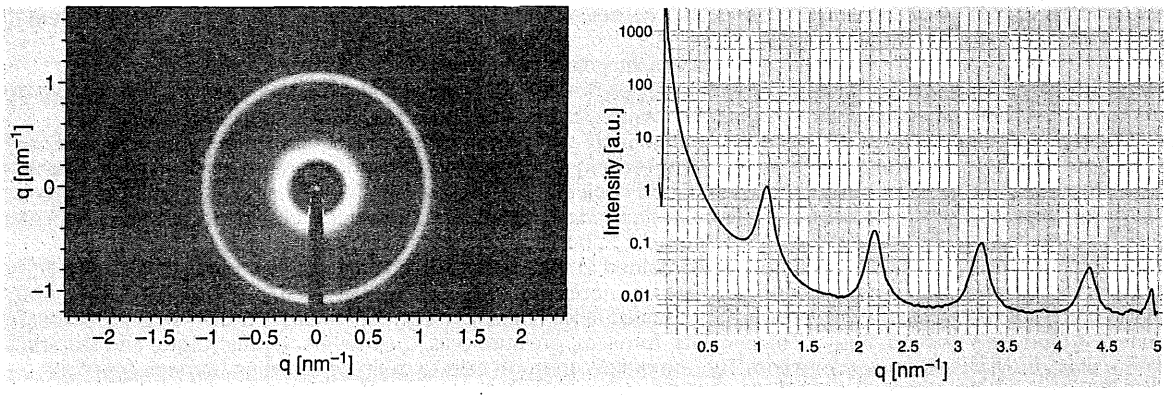

Figure 6. Two representations of the SAS (in this case, SAXS) from the same sample of polycrystalline silver behenate.

6.3.2 INTENSITY CALIBRATION

Intensity calibration relies on the fact that for some types of material, $I(q = 0)$ can be related to molecular parameters that can be calculated with reliability or determined using other techniques, for example, the molecular weight of a polymer, the surface area-to-volume ratio of a porous solid, or the second virial coefficient or isothermal compressibility of a pure liquid. However, because an SAS instrument does not physically collect data at $q = 0$, an extrapolation is required. Extrapolation is only possible, with the necessary precision, if the scattering function for the chosen calibrant has a definitive form. Commonly used intensity calibrants include water (SAXS; *10*), Lupolen (SAXS), blends of hydrogenated and perdeuterated homopolymers (SANS; *11,18*), porous solids (SAXS/SANS; *18*), glassy (vitreous) carbon (SAXS/SANS; *12,18*), and vanadium (SANS).

6.4 Sample Quality

As in any experiment, the best results will be obtained using carefully prepared samples. In particular, because the SAS from an object scales as the sixth power of its size, the presence of even a relatively few large objects in a sample can disproportionately interfere with the quality of the data, all else being equal. SALS is particularly affected by the presence of large particles because of the aforementioned difficulties in RI matching. For this reason, all solvents used for SALS experiments should be rigorously filtered before use to exclude the presence of dust particles. SAXS and SANS (but not USAXS/USANS) are much less affected by dust because they probe shorter length scales (dust particles are micron-sized). However, the presence of unwanted, unintended, or unexpected aggregation of components of the sample can be just as troublesome. Prescreening for aggregation in biological samples destined for SAXS or SANS instruments (for example, by DLS) should be considered *de rigour*. Poor contrast in a sample can also be problematic, although in some instances (for example, in SANS contrast matching experiments), it is unavoidable.

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6.5 Sample Concentration

Although the magnitude of $I(q)$, and the precision with which it is measured, are both generally improved by using more concentrated samples, this comes with disadvantages as well, i.e., lower transmissions (see *6.9 Sample Transmission*) and the possible introduction of atomic pair correlations manifesting themselves in an $S(q)$ contribution to the scattering function. Whether this is an issue depends on the sample being studied and the intended method of data analysis. Some relatively simple modeling of the scattering before the experiment may help to establish the viable concentration envelope.

Of course, the converse is also true, and very dilute samples will likely suffer from a poor signal-to-noise ratio and necessitate longer experimental counting times, although this longer experimental time might be mitigated by the use of a high-flux instrument. However, as an illustration, most SAXS/SANS instruments can handle protein/enzyme solutions at concentrations as low as $\Phi \sim 0.1\%$ (1 mg/mL), whereas $S(q)$ is not usually a concern in a colloidal dispersion at $\Phi < 5\%$. For investigations of $S(q)$, relevantly higher concentrations are used.

6.6 Sample Containers

Unless one is working with a self-supporting sample, the sample will need to be contained. The problem with sample containers is that they also scatter, so the material from which they are constructed is an important consideration. Suitable container window materials combine the mechanical strength and chemical resistance with high transmittance and scattering features, which do not interfere with those of the sample. As such, multiple window materials may be necessary to cover the entire range over which a sample is to be measured.

For SALS and SAXS, it is quite common to use glass sample containers. Glass is composed only of light atoms (an important consideration for SAXS), and its transparency is as useful for observing the sample as it is a prerequisite for SALS. However, because glass contains boron (a neutron absorber) it is not suitable for SANS.

Amorphous synthetic quartz (SiO2, also called fused silica) is a good choice of material for SALS, SAXS, and SANS. It can be cut, polished, and fabricated and is also transparent and only composed of light atoms; however, it is boron-free. It also has good thermo-mechanical stability over an experimentally useful range of temperatures and is chemically inert to all but concentrated bases and hydrofluoric acid. Material tradenames include Spectrosil and Suprasil, but in principle anything suitable for far-UV (not IR) spectrophotometry should suffice.

For SAXS, it is possible to utilize sample containers constructed from amorphous engineering polymers such as poly(carbonate) (PC), poly(etheretherketone) (PEEK), or poly(imide) (Kapton). Poly(amides) (nylons) are not suitable because they are semi-crystalline. All hydrogenous polymers are wholly unsuitable for use as SANS sample containers.

The greater penetrating ability of neutrons means that SANS sample containers can be constructed from metals. Pure metals should be used where possible (as SANS is an excellent technique for probing the nanostructure of metal alloys), or when a prior assessment of suitability has been conducted. Good choices of metals for use with neutrons are aluminum, titanium, vanadium, and copper. These metals can activate on prolonged exposure to neutron radiation but not to levels that make them impractical to use. Beryllium, being of low atomic number, is however a practical choice for SAXS.

In most experiments, the sample will simply be contained in the quiescent state in a capillary or cuvette. However, it is important to understand that quiescent conditions are not necessary. Indeed, the ability of SAS to probe a sample under changing conditions (pH, temperature, pressure) or applied fields (electric, magnetic, shear) is usually where the greatest information is ultimately derived. The use of cryostats, furnaces, pressure cells, rheometers, tensile stages, and stop-flow cells with SAXS/SANS instruments is very common. The use of flow-through cells to mitigate radiation damage (see *6.11 Radiation Damage*) is very common on high-flux SAXS instruments.

Sample containers will typically have path lengths ranging from 0.1–2 mm (SAXS) to 1–5 mm (SANS) to 10 mm or more (SALS). Multiplying this value by the incident beam size—a few micrometers (SAXS) to a few millimeters (SALS/SANS)—reveals that the required sample volume is rarely more than a couple of milliliters, and potentially less.

6.7 Backgrounds

In the same way that the sample container contributes to the measured scattering, so does the matrix containing the sample, for example, the dispersion medium. For this reason, it is typical to measure the scattering from the pure matrix, in the same or an identical sample container under the same experimental conditions, and then to subtract this matrix scattering from the sample scattering. This procedure should also correct for other sources of "sample-independent" background (for example, the container, vacuum windows, detector response, etc.).

Difficulties can arise if the sample component of interest occupies a substantial proportion of the overall sample volume because subtracting 100% of the matrix may no longer be appropriate, leading to over-correction of the data. In these instances, a volume fraction-weighted background correction may be appropriate.

Arguably, the most insidious form of "sample-independent" background is multiple scattering (discussed in *6.10 Multiple Scattering*).

6.8 Counting Times

Experimental measuring times for an SAS experiment may vary from a fraction of a second (synchrotron-SAXS) to many minutes or hours (SANS). However, because SAS measurements are Poisson counting processes, to halve the statistical uncertainty on an $I(q)$ point requires four times as much data. This calculation is further complicated by the fact that when a background is subtracted, the statistical errors on the sample and background are added in quadrature, as shown here:

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$$Error_{sample-background} = \sqrt{\left\{\left(Error_{sample}\right)^2 + \left(Error_{matrix}\right)^2\right\}}$$

Thus the final data quality may not be apparent until after the experiment has finished. This means that invariably one ends up making a judgment call about the statistical quality of the data before moving on to the next sample. Therefore, counting times are often based on previous experience or simple trial-and-error, or a combination of both. A good rule of thumb is to measure each background for the same number of photons/neutrons as the sample it is intended to correct.

6.9 Sample Transmission

The sample transmission, T_r, is a dimensionless number ranging between 0 and 1 and is a measure of the balance between the scattering power of the sample and absorption as described by the Beer–Lambert Law:

$$T_r = exp(-\mu t) = 1 - A$$

where t is the pathlength (in most instances, the thickness) of the sample, μ is an absorption coefficient, and A is the absorbance; μ is dependent on the type of radiation in use.

Despite the simple appearance of this expression, accurately calculating T_r for a sample is fraught with difficulties because μ may be unknown or difficult to determine. The best course of action is to measure T_r at the time the SAS is recorded, simultaneously, if possible. Note that in TOF–SANS, T_r is a function of wavelength.

A good rule of thumb is to keep $T_r > 0.37$ (i.e., $t < 1/\mu$), although this will have consequences for multiple scattering (see *6.10 Multiple Scattering*).

6.10 Multiple Scattering

Multiple scattering is where a photon/neutron that has been scattered once by the sample is scattered again (possibly several times). Multiple scattering should be avoided, if possible, because a routine method for correcting SAS data for multiple scattering has not been developed, except in some limited circumstances. The essence of the problem is that the history of multiply scattered photons/neutrons is unknown, and thus one cannot know whether or not to use them.

It is, however, quite straightforward to estimate the extent of multiple scattering from a sample. To a first approximation, the average number of times a photon/neutron has been scattered, τ, is:

$$\tau = \mu t = -\ln T_r \propto t\lambda^2$$

Thus, even for $T_r = 0.9$ there is a 10% probability of multiple scattering. By $T_r = 0.5$ that probability has risen to almost 70%.

If multiple scattering is suspected, two strategies for mitigating its effects are to thin the sample and/or use shorter wavelengths and then re-measure and look for differences.

6.11 Radiation Damage

An important consideration during SAXS, particularly synchrotron-SAXS, experiments is that the X-ray beam can be extremely destructive to some samples if mitigating strategies are not deployed, such as the use of flow-through cells or cryogenic cooling. This is because the energy of the X-ray photons [for example, 8047 eV at the copper (Cu) (Kα) wavelength of 0.154 nm] is far greater than the strength of the bonds holding the sample together (for example, the C–C bond energy is just 4 eV), and hydrogen bonds are weaker still. Even visible photons from a modest blue laser can cause sample degradation if left to irradiate the sample for long enough. Conversely, the energy of neutrons of equivalent wavelengths are orders of magnitude lower (for example, a mere 0.034 eV at a wavelength of 0.154 nm), meaning neutrons are a truly non-destructive probe, especially of sensitive biological material.

6.12 Sample Deuteration

Although selective deuteration is a valuable experimental tool for extracting maximum information from a SANS experiment, it is unfortunately not without complications. The slightly larger atomic volume of deuterium, differences in the polarizability of C–H bonds and C–D bonds, and differences in the strength of O…H and O…D hydrogen bonds all contribute to subtle modifications of the underlying thermodynamics in the sample which can, and do, affect the phase behavior. For example, cloud points, critical micelle concentration, micelle aggregation numbers, and theta temperatures have all been known to change (sometimes in opposite directions depending on whether the solute or the solvent is deuterated). These challenges should be considered when designing a SANS experiment.

7. DATA PROCESSING

SAS data processing has two steps:

1. Data reduction: The transformation of the as-measured instrument-dependent data into portable instrument-independent data (ideally in absolute units on an absolute scale)

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2. Data analysis: The interpretation of the instrument-independent data to extract physical information characterizing the sample

7.1 Data Reduction

Data reduction procedures, and the factors for which they are corrected, will vary from one SAS instrument to the next. In the case of SANS, these procedures can even vary between reactor-SANS instruments and TOF-SANS instruments. However, every SAS instrument manufacturer or large facility will provide the necessary software to correctly reduce the data collected from their instrument(s). This software is typically graphical user interface (GUI)-driven (although scripts are still used) and will offer both "novice" and "expert" modes of use, for either "manual"/"single" or "automated"/"batch" data reductions.

In general, the data reduction process will include most or all of the following steps:

1. Correct for the detector efficiency, dark current, dead time, and spatial linearity (flatness)
2. Calculate the q values of each detector pixel
3. Integrate the recorded counts at a given q within some region of interest (azimuthal angle, sector, etc.)
4. Normalize for the incident flux or quantum efficiency of the source
5. Correct for the sample transmission (absorbance correction)
6. Correct for the volume of sample illuminated (beam area × sample thickness)
7. Apply an intensity calibration factor
8. Convert the intensity scale into absolute units

This process is repeated for every sample measured, and for any backgrounds measured. The appropriate background is then subtracted from a sample to yield the final, fully reduced SAS data set.

7.2. Data Analysis

SAS data analysis for a single sample may use one or more of the following methodologies:

- Model-fitting/curve-fitting methods:
  - Involve optimized fitting of analytical functions for $P(q)$ and/or $S(q)$ to the experimental $I(q)$ data, for example, to determine generic shape information (sphere, cylinder, coil, etc.), size (radius, radius-of-gyration, cross-sectional radius, etc.), and interaction potentials (hard sphere, Percus–Yervick, etc.) (see *Figure 7*)
  - Applicable to all sample types
  - Computationally straightforward

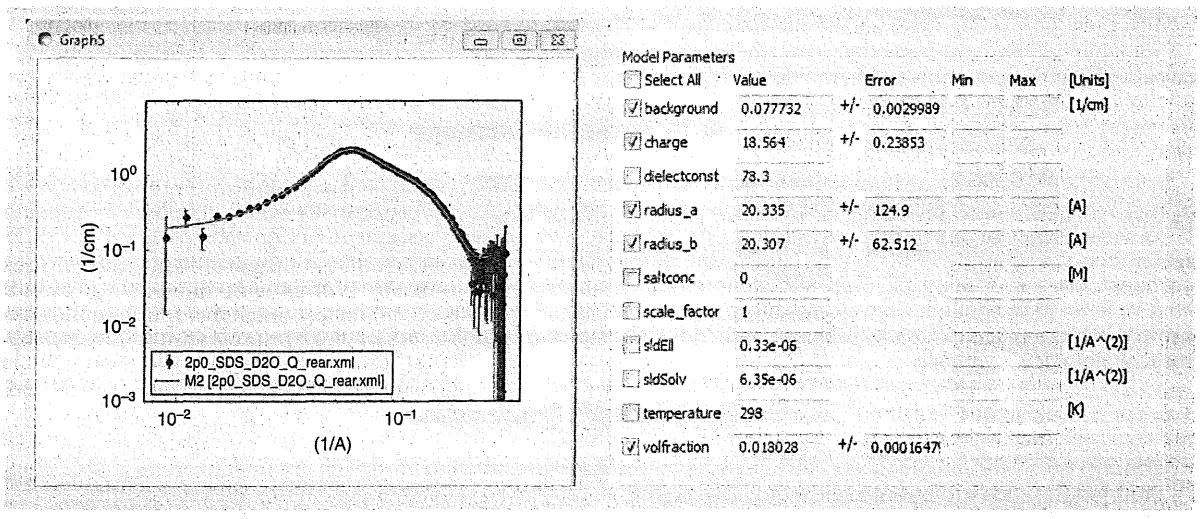

Figure 7. Left: Fit of a model calculation (line) to the SANS from a 2% (w/w) solution of surfactant sodium dodecyl sulfate (SDS) in D_2O (shaded circles). At this concentration, above the critical micelle concentration (CMC), the system comprises electrostatically interacting, near-spherical, charged micelles. Right: The model parameter set, illustrating the level of sophistication that this approach can bring. Data and figures courtesy of Doucet et al., 2017 (*13*).

- Real-space methods:
  - Involve mathematical transformation of the experimental $I(q)$ data from reciprocal-space to real-space, for example, to obtain density correlation functions ($\Gamma(r)$), distance distribution functions [pair-distance distribution function (PDDF) or $P(r)$], or scattering length/segment density profiles [$\rho(r)$] (see *Figure 8*)
  - Applicable to all sample types
  - Computationally straightforward

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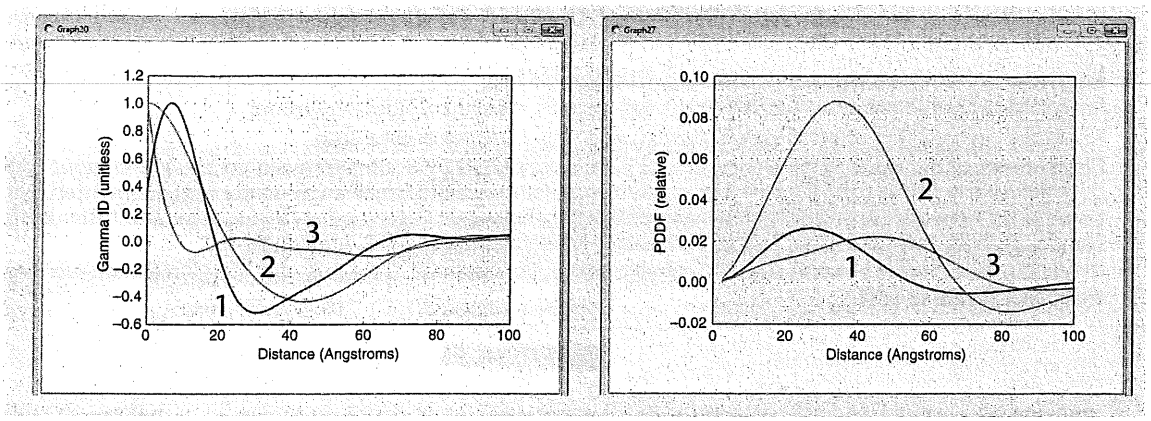

Figure 8. Two representations of the structure of a surfactant-stabilized oil-in-water emulsion system obtained from the SANS at three different scattering contrasts: "core" (line 1: where the scattering arises from the oil droplets only); "drop" (line 2: where the scattering arises from the oil cores and the surfactant layers); and "shell" (line 3: where the scattering arises from the surfactant layers only). For this system, the model-fitting approach suggests relatively monodisperse spherical objects of 25 Å radius with a 6 Å thick surfactant shell. Left: The one-dimensional density correlation functions. Right: The pair-distance distribution functions. Data and figures courtesy of Doucet et al., 2017 (*13*).

- $\Gamma(r)$ depicts the average separation of regions of similar scattering-length density in the sample. The minima and maxima in the function represent structural boundaries, for example, the edge of the object:

$$\Gamma(r) = \frac{1}{Q^*}\int_0^\infty I(q)q^2\cos(qr)dq$$

where the "scattering invariant" (also called the "total scattering") is given by:

$$Q^* = \int_0^\infty I(q)q^2dq$$

- The PDDF depicts the relative number of instances that two points in each scattering object are separated by a given distance, *r*. It is a function of distance only and is not a three-dimensional representation of the structure. The more symmetric the curve, the more rotationally symmetric the shape of the scattering object. Basic shapes like spheres, rods, and disks produce distinct distributions.

$$PDDF = \frac{1}{2\pi^2}\int_0^\infty I(q)qr\sin(qr)dq$$

- Ab-initio methods:
  - Involve the iterative creation of complex-shape envelopes, for example, from assemblies of homogeneous spheres ordered on a lattice or using spherical harmonic functions, the computed scattering of which is then compared with the experimental $I(q)$ data (*14*,*15*)
  - Are particularly useful for approximating the tertiary structure of biological molecules, but can also be applied to self-assembled soft matter systems like micelles
  - Do not take into account the chemical physics of the sample (e.g., bond lengths, bond angles, packing fractions, electrostatics)
  - Are computationally modest
- Simulation methods:
  - Involve the iterative manipulation of atomistic structures, for example, as derived from X-ray or high-resolution NMR crystal structures, by Boltzman-weighted Monte Carlo/Molecular Dynamics methods, the computed scattering of which is then compared with the experimental $I(q)$ data (*16*,*17*)
  - Are particularly useful for determining the tertiary structure of biological molecules
  - Take into account the chemical physics of the sample
  - Are computationally expensive, but can be used with coarse-graining methods

As SAS data analysis procedures become ever more sophisticated, so too does the degree of detail about the sample that they can return. However, one must never take this information in isolation; always look to cross-validate it with information derived from alternative techniques or the scientific literature. For example, model-fitting a large number of parameters without a priori information can result in "local" rather than "global" solutions. And PDDF, ab initio, and simulation methods work best when they can be constrained (e.g., to a known radius-of-gyration).

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8. ADDITIONAL SOURCES OF INFORMATION

- For further information about SAS, visit http://smallangle.org.
- For further information about synchrotron X-ray sources, visit http://lightsources.org.
- For further information about neutron sources, visit http://neutronsources.org.
- The interests of the world-wide SAS community are represented by a subcommission of the International Union of Crystallography (IUCr) (http://www.iucr.org/resources/commissions/small-angle-scattering) augmented by the community network CanSAS (http://www.cansas.org/). Together, these bodies advocate for and foster best practice, standardization, and data interchange, particularly where SAXS and SANS are concerned.
- The IUCr also operates a moderated SAS-specific email discussion list. To join it, visit http://mailman.iucr.org/cgi-bin/mailman/listinfo/sa_scat.

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▲ (USP 1-Dec-2019)

Add the following:

▲⟨1467⟩ RESIDUAL SOLVENTS—VERIFICATION OF COMPENDIAL PROCEDURES AND VALIDATION OF ALTERNATIVE PROCEDURES

The procedures defined in *Residual Solvents* ⟨467⟩ have been validated for Class 1 and 2 solvents over the range of 50%–150% of the concentrations described in the standard preparation for procedures A and B in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents.*

The suitability of these residual solvent procedures must be verified under actual conditions of use. When using the procedures for testing residual solvents described in ⟨467⟩, verification can be accomplished using the recommendations below. There are separate recommendations for limit and quantitative procedures in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents.*

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Alternative procedures include different analytical procedures from those described in ⟨467⟩, as well as modifications to the procedures described therein that go beyond the stated validation of the method (e.g., different concentrations or other analytes or variations to chromatographic conditions beyond those permitted by *Chromatography* ⟨621⟩). Alternative procedures are permitted (see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*), provided that they are properly validated. A risk-based approach may be appropriate to determine the degree and extent of the verification or validation process to assure the fitness for purpose of the procedure. Recommendations for validation of alternative procedures (limit and quantitative tests) are described in *Validation of Alternative Procedures*. A summary of the requirements is shown in *Table 1*.

Table 1. Summary of Verification and Validation Requirements

	Verification of Compendial Procedures		Validation of Alternative Procedures	
Validation/Verification Characteristics	Limit Test Methods	Quantitative Methods	Limit Test Methods	Quantitative Methods
Specificity	Yes	Yes	Yes	Yes
Detection limit	Yes[a]	No	Yes	No
Quantitation limit[b]	No	Yes[a]	No	Yes
Accuracy	No	Yes	No	Yes
Precision/repeatability	No	Yes	No	Yes
Linearity	No	No	No	Yes
Range	No	Demonstrated by accuracy and precision	No	Demonstrated by accuracy and precision
Intermediate precision	No	No	No	Yes
Solution stability[c]	Yes	Yes	Yes	Yes
Robustness[d]	No	No	Yes[e]	Yes[e]

[a] System suitability may be used instead to demonstrate sensitivity.
[b] In quantitative tests, quantitation limit may be demonstrated by accuracy and precision determination.
[c] Solution stability should be determined for the timeline of the test.
[d] Evaluation of robustness should be considered during the development phase and depends on the type of procedure under study.
[e] The "Yes" quotation here is intended to emphasize the importance of assessing the robustness of the procedure before the implementation.

VERIFICATION OF COMPENDIAL PROCEDURES

Limit Procedures: Procedure A and Procedure B

The analytical characteristics to be verified include specificity, detectability, and solution stability.

VERIFICATION WHEN SOLVENTS LIKELY TO BE PRESENT (LTBP) ARE NOT KNOWN

Samples

Prepare a reagent blank.

Prepare *Standard solutions* as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

Prepare the *Spiked sample solution* as described in *Class 1 System suitability solution* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

Specificity

Recommended acceptance criteria: An appropriate blank should be injected to assure the lack of a significant interference. A significant interference is one producing a deviation in the fitness for purpose of the procedure that affects precision and/or accuracy. The procedure must be able to separate acetonitrile and methylene chloride (*Procedure A* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*), or acetonitrile and *cis*-dichloroethene (*Procedure B* in *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*) with a resolution of NLT 1.0.

Detection Limit (see *Table 1, Footnote a*)

Recommended acceptance criteria: The mean signal-to-noise ratio for each solvent in the *Standard solution* and *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations from a single preparation is NLT 3.

Solution Stability

Recommended acceptance criteria: Detection limit should meet the requirements throughout the testing period.

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VERIFICATION WHEN SOLVENTS LTBP ARE KNOWN

Samples

Prepare a reagent blank.

Prepare *Standard solution(s)* and *Sample solution(s)* as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* depending on the specific sample solubility using only those solvents likely to be present (LTBP).

Prepare *Spiked sample solution(s)* as described in *Quantitative Procedures: Procedure C* for solvents LTBP [corrected for native (original) solvent content].

Specificity

Recommended acceptance criteria: The reagent blank does not produce any significant interference with any of the peaks from solvents LTBP.

The procedure must be able to separate each of the solvents in the *Standard solution(s)* from each other and from other peaks in the *Spiked sample solution* with a resolution of NLT 1.0. If the resolution between any pair of peaks is less than 1.5, then verification must demonstrate that the method is suitable for its intended use.

If the solvents present in the *Standard solution(s)* are not separated with a resolution of NLT 1.0 when using the *Chromatographic System* of *Procedure A*, then *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* should be used as confirmatory.

Detection Limit

Recommended acceptance criteria: The mean signal-to-noise ratio for each solvent in the *Standard solution* and the *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations from a single preparation is NLT 3.

Solution Stability

Recommended acceptance criteria (not required if only running fresh solutions): Detection limit for limit tests and quantitation limit for quantitative tests should be met throughout the testing period.

Quantitative Procedures: Procedure C

[NOTE—When performing *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.5 Quantitative Tests—Procedure C*, solvents LTBP are typically known, either based on results from *Procedure A* or *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*, or based on available knowledge. The analytical characteristics to be verified include specificity, accuracy (which addresses quantitation limit), range, repeatability, and solution stability.]

Samples Prepare a reagent blank.

Standard stock solution: Prepare a solution containing each solvent LTBP or each peak identified and verified by *Procedure A* and *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*, with a concentration as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.5 Quantitative Tests—Procedure C* for water-soluble articles or water-insoluble articles, as appropriate.

Sample stock solution: Prepare as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

Spiked sample solution: Prepare as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.5 Quantitative Tests—Procedure C*, using the *Standard stock solution*.

Spiked sample solutions A, B, C, etc.: Prepare *Spiked sample solutions* with the sample matrix and spiked with each solvent LTBP or identified and verified by *Procedure A* and *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*, in triplicate, at NLT 3 levels covering the range of interest or at least 50%–150% of the corresponding concentration of the *Standard solution* as described in *Procedure A* in *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

[NOTE—The *Spiked sample solution* may be used as one of the solutions.]

Specificity

Recommended acceptance criteria: The procedure must be able to separate each of the solvents in the *Standard solution(s)* from each other and from other peaks in the *Spiked sample solution* with a resolution of NLT 1.0. If the resolution between any pair of peaks is less than 1.5, then verification must demonstrate that the method is suitable for its intended use.

If the solvents present in the *Standard solution(s)* are not separated with a resolution of NLT 1.0, then chromatography described in *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* should be used as confirmatory.

Quantitation Limit

Recommended acceptance criteria: The mean signal-to-noise ratio for each solvent in the *Standard solution* and the *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations is NLT 10, or the *Quantitation Limit* may be demonstrated by *Accuracy* and *Precision*.

Accuracy

Recommended acceptance criteria: The mean recovery for *Spiked sample solutions A, B,* and *C, etc.*, when calculated relative to the *Spiked sample solution*, is 80%–120% of the expected theoretical amount.

[NOTE—Recoveries should be corrected for native (original) content of any solvent under test.]

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Precision/Repeatability Use at least six independent *Spiked sample solution* preparations from the same lot, prepared as described in the *Spiked sample solution* in the *Accuracy* test above, or use nine independent preparations as described in *Spiked sample solutions A, B, C, etc.*
Recommended acceptance criteria: Relative standard deviation is NMT 20% for each solvent present.
Solution Stability: Demonstrate acceptable solution stability for the period of time to run the test.
Recommended acceptance criteria: NMT 20% change in solvent(s) content compared to the initial time point

VALIDATION OF ALTERNATIVE PROCEDURES

Chromatographic alternative procedures should meet the acceptance criteria for the analytical performance characteristics shown below. When non-chromatographic alternative procedures are validated, the analytical performance characteristics listed should be addressed, although, it may be appropriate to apply other analytical performance characteristics. For more information, refer to *Validation of Compendial Procedures* ⟨1225⟩.

Limit Procedures

Analytical characteristics to be validated are specificity, detection limit, and solution stability. The same criteria used for *Verification when solvents LTBP are known*, as described above, may be used.

Quantitative Procedures

Analytical characteristics to be validated are specificity, linearity and range, quantitation limit, accuracy, repeatability, intermediate precision, and solution stability.
Samples Prepare a reagent blank.
Spiked sample solutions P, Q, R, etc.: Prepare *Spiked sample solutions* with the sample matrix and spiked with each solvent LTBP at NLT 5 levels covering the range of interest. [NOTE—Results should be corrected for native (original) content of any solvent under test.]
Specificity
Recommended acceptance criteria: The analytical procedure must have the ability to assess unequivocally the analytes of interest in the presence of the components expected to be present.
Linearity and Range
Recommended acceptance criteria: Perform linear regression analysis on the results for *Spiked sample solutions P, Q, R, etc.* The coefficient of determination, r^2, is NLT 0.90.
Quantitation Limit
Recommended acceptance criteria: The mean signal-to-noise ratio for each solvent in the standard solution and the *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations is NLT 10. The *Quantitation Limit* may also be demonstrated by *Accuracy* and *Precision.*
Accuracy
Recommended acceptance criteria: The mean recovery for each *Spiked sample solution* should be 80%–120%. [NOTE—Recoveries should be corrected for native (original) content of any solvent under test.]
Precision/Repeatability: Prepare at least six independent *Spiked sample solution* preparations from the same lot.
Recommended acceptance criteria: Relative standard deviation is NMT 20% for each solvent present.
Precision/Intermediate Precision: Perform the *Repeatability* test over at least two independent events, e.g., on different days, and/or using different instruments and/or analysts.
Recommended acceptance criteria: Testing for intermediate precision should demonstrate that the method is suitable for its intended use.
Solution Stability: Standard solutions and *Spiked sample solutions* should be stable throughout the testing period.
Recommended acceptance criteria: NMT 20% variation ▲2S (USP41)

⟨1601⟩ PRODUCTS FOR NEBULIZATION—CHARACTERIZATION TESTS

Products used for nebulization and intended for pulmonary delivery are characterized using the following tests:

- *Drug Substance Delivery Rate and Total Drug Substance Delivered*;
- *Aerodynamic Assessment of Nebulized Aerosols*.

These tests standardize the approach to the assessment of the dose that would be delivered to a patient but are not intended to provide assessment of the nebulizer device itself.[1] The mass rather than the number-weighted size distribution is more appropriate to evaluate product performance. Drug substance mass as a function of aerodynamic diameter is more indicative of therapeutic effect within the respiratory tract.

[1] European Standard 13544-1:2001. *Respiratory Therapy Equipment*. Part 1: Nebulizing systems and their components. European Committee for Standardization. Brussels, Belgium. 2001.

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DRUG SUBSTANCE DELIVERY RATE AND TOTAL DRUG SUBSTANCE DELIVERED

These tests are performed to assess the rate of delivery to the patient and the total drug substance delivered to a patient using standardized conditions of volumetric flow rate. Breath-enhanced and breath-actuated nebulizers should be evaluated by a breathing simulator because the output of these types of device is highly dependent on inhalation flow rate. The methodology below describes the use of a standard breathing pattern defined for adults. Should a particular product for nebulization be indicated only for pediatric, i.e., neonate, infant, or child use, then pediatric breathing pattern(s) must be used.[2] Breathing patterns are used, rather than continuous flow rates, to provide a more appropriate measure of the mass of drug substance that would be delivered to patients.

Drug substance delivery rate and total drug substance delivered are appropriate characteristics because they allow the mass delivered to be characterized in a standard way regardless of the nebulizer used. Accordingly, the test methodology described below (a) measures the mass of drug substance delivered in the first period (typically 1 min) consequently giving an assessment of drug substance delivery rate and (b) captures the total drug substance mass delivered.

Apparatus

BREATHING SIMULATOR

A commercially available breathing simulator that is able to generate the breathing profiles specified in *Table 1* is used for the test. The breathing profile indicated for adults is used unless the medicinal product is specifically intended for use in pediatrics, when alternate patterns should be used, as indicated in *Table 1*.

Table 1. Breathing Simulator Specification

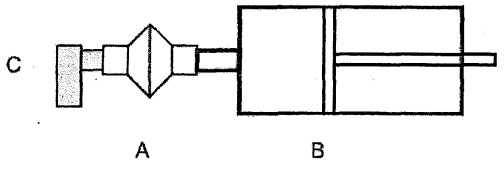
Item	Specification			
	Adult	Neonate	Infant	Child
Total volume	500 mL	25 mL	50 mL	155 mL
Frequency	15 cycles/min	40 cycles/min	30 cycles/min	25 cycles/min
Waveform	sinusoidal	sinusoidal	sinusoidal	sinusoidal
Inhalation:exhalation ratio	1:1	1:3	1:3	1:2

FILTER SYSTEM

A suitably validated low-resistance filter, capable of quantitatively collecting the aerosol and enabling recovery of the drug substance with an appropriate solvent, is used for the test. The dead volume of the filter casing does not exceed 10% of the tidal volume used in the breath simulation.

Procedure

Attach the filter (contained in the filter holder) (A) to the breath simulator (B) according to *Figure 1*. Fill the nebulizer (C) with the volume of the drug product as specified in the patient instructions. Attach the mouthpiece of the nebulizer to the inhalation filter using a mouthpiece adapter if required, ensuring that connections are airtight. Position the nebulizer in the same orientation as intended for use. This may require tilting the breathing simulator and filter holder. Set the breathing simulator to generate the specified breathing pattern.



A. inhalation filter and filter holder B. breathing simulator C. nebulizer

Figure 1. Experimental Set-Up for Breathing Simulator Testing.

Start the breathing simulator and at the beginning of an inhalation cycle, start the nebulizer. Operate the nebulizer for a defined initial time period. The length of the time interval ensures that sufficient drug substance is deposited on the inhalation

[2] Suitable breathing patterns for pediatric use may be found, for example, in Canadian Standard CAN/CSA/Z264.1-02:2002, *Spacers and Holding Chambers for Use with Metered Dose Inhalers*. Canadian Standards Association, Mississauga, Canada 2002.

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filter for quantitative analysis. A time of 60 ± 1 s typically enables direct determination of the drug substance delivery rate. The time chosen, usually 60 ± 1 s, must allow sufficient drug substance deposition on the inhalation filter to allow quantitative analysis. If the quantity of drug substance deposited on the inhalation filter in 60 s is insufficient for this analysis, the length of the time interval for aerosol collection can be increased. If the filter is soaked with the product, this time can be decreased. At the end of this initial period, stop the nebulizer.

Place a fresh filter and filter holder in position and continue until nebulization ceases. Interrupt nebulization and exchange filters, if necessary, to avoid filter saturation.

Results

Using a suitable method of analysis, determine the mass of drug substance collected on the filters and filter holders during each time interval. Determine the drug substance delivery rate by dividing the mass of drug substance collected on the first inhalation filter by the time interval used for collection. Determine the total mass of drug substance delivered by summing the mass of drug substance collected on all inhalation filters.

Change to read:

AERODYNAMIC ASSESSMENT OF NEBULIZED AEROSOLS

Nebulized products need to be size-characterized at flow rates lower than the range that is typically used for powder inhalers and metered-dose inhalers. The CEN standard recommends a flow rate of 15 L/min because this value represents a good approximation to the mid-inhalation flow rate achievable by a healthy adult breathing at 500 mL tidal volume.

Although low-angle laser light-scattering instruments (laser diffractometers) can provide rapid size-distribution measurements of nebulizer-generated aerosols, these techniques do not detect the drug substance. Rather, they measure the size distribution of the droplets irrespective of their content. This may not be a problem with homogeneous solutions, but it can result in significant error if the product to be nebulized is a suspension, or if droplet evaporation is significant, as can be the case with certain nebulizer types. Cascade impactors enable the aerosol to be characterized unambiguously in terms of the mass of drug substance as a function of aerodynamic diameter. Laser diffraction may be used if validated against a cascade impaction method.

Apparatus 5 (see ▲general chapter *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩▲ (ERR 1-Jun-2018)), a cascade impactor, has been calibrated at 15 L/min specifically to meet the recommendation of the CEN Standard and is therefore used for this test.[3] Determining mass balance in the same way as for powder inhalers and metered-dose inhalers is not straightforward because the dose is being captured as a continuous output and hence is not included. Recovery experiments must be performed as part of method development and validation. Control of evaporation of droplets produced by nebulizers may be critical to avoid bias in the droplet size assessment process. Evaporation can be minimized by cooling the impactor to a temperature of about 5°, typically achieved by cooling the impactor in a refrigerator for about 90 min.

Typically, at least after each day of use, the apparatus must be fully cleaned, including the inter-stage passageways, because of the greater risk of corrosion caused by the condensation/accumulation of saline-containing droplets on inter-stage metalwork associated with cooling the impactor. All surfaces of the apparatus should be dried after each test, e.g, with compressed air. [NOTE—The micro-orifice collector (MOC) should not be dried with compressed air.]

Apparatus

A detailed description of Apparatus 5 and the induction port is contained in ⟨601⟩, and includes details of critical dimensions and the qualification process for the impactor (stage mensuration).

A back-up filter in addition to the MOC must be used to ensure quantitative recovery of drug substance from the nebulized aerosol at the specified flow rate of 15 L/min. The filter is located below the MOC (internal filter option), or a filter in holder external to the impactor is used to capture any fine droplets that pass beyond the last size fractionating stage. A pre-separator is not used for testing nebulizer-generated aerosols.

Method Validation

IMPACTOR STAGE OVERLOADING

During method development and validation, confirm that the volume of liquid sampled from the nebulizer does not overload the impactor. Visual inspection of the collection surfaces on stages collecting most of the droplets may reveal streaking if overloading has occurred. This phenomenon is usually also associated with an increase in mass of drug substance collected on the final stage and back-up filter. Reducing the sampling period (T_o) is the most effective way to avoid overloading in any given system, balancing overloading with analytical sensitivity.

RE-ENTRAINMENT

Droplet bounce and re-entrainment are less likely with nebulizer-produced droplets than with solid particles from inhalers, thus coating would not normally be required.

[3] Marple VA, Olson BA, Santhanakrishnan K, *et al.* Next generation pharmaceutical impactor: A new impactor for pharmaceutical inhaler testing. Part III: Extension of archival calibration to 15 L/min. *J Aerosol Med* 2004; 17(4):335–343.

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Procedure

Pre-cool the assembled impactor and induction port in a refrigerator (set at about 5°) for not less than 90 min, and start the determination within about 5 min of impactor removal from the refrigerator. Other methods that maintain the impactor at a constant temperature (e.g, use of a cooling cabinet) can also be employed when validated.

Set up the nebulizer with a supply of driving gas (usually air or oxygen), or use a compressor at the pressure and flow rate specified by the manufacturer of the nebulizer. Ensure that the gas supply line does not become detached from the nebulizer when under pressure. Fill the nebulizer with the volume of the medicinal product as specified in the patient instructions.

Remove the impactor from the refrigerator. Attach the induction port to the impactor, and connect the outlet of the impactor/external filter to a vacuum source that is capable of withdrawing air through the system at 15 L/min, as specified in *Figure 2*. Turn on the flow through the impactor.

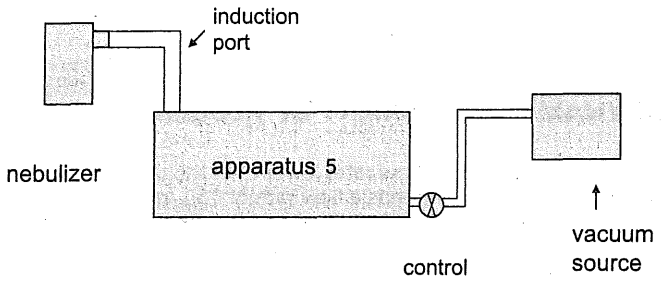

Figure 2. Apparatus 5 for Measuring the Size Distribution of Products for Nebulization.

Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve located between the impactor and the vacuum source to achieve a steady flow through the system at 15 L/min (±5%). Remove the flow meter.

Position the nebulizer in the same orientation as intended for use, then attach the mouthpiece of the nebulizer to the induction port, using a mouthpiece adapter if required. Switch on the flow/compressor for the nebulizer. Sample for a predetermined time (T_o). Once determined, this time (T_o) must be defined and used in the analytical method for a particular drug product to ensure that mass fraction data can be compared. At the end of the sampling period, switch off the driving gas flow/compressor to the nebulizer, remove the nebulizer from the induction port, and switch off the flow from the vacuum source to the impactor. Dismantle the impactor and, using a suitable method of analysis, determine the mass of drug substance collected in the induction port on each stage and on the back-up filter as described for Apparatus 5 (see ⟨601⟩). Add the mass of drug substance collected in the MOC to that deposited on the back-up filter/external filter and treat as a single sample for the purpose of subsequent calculations.

Calculate the fine particle mass of the drug substance based on the predetermined time (T_o). Calculate the mass fraction ($F_{m,comp}$) of the drug substance deposited on each component of the impactor, commencing with the induction port and proceeding in order through the impactor, using the following expression:

$$F_{m,comp} = m_{comp}/M$$

m_{comp} = mass associated with the component under evaluation;

M = total mass collected by the system.

Present $F_{m,comp}$ in order of location within the measurement equipment, beginning at the induction port and ending with the back-up filter of the impactor (see *Figure 3*). $F_{m,comp}$ values for adjacent stages of the impactor may be combined in order to report the mass fraction collected on a group of stages as a single value.

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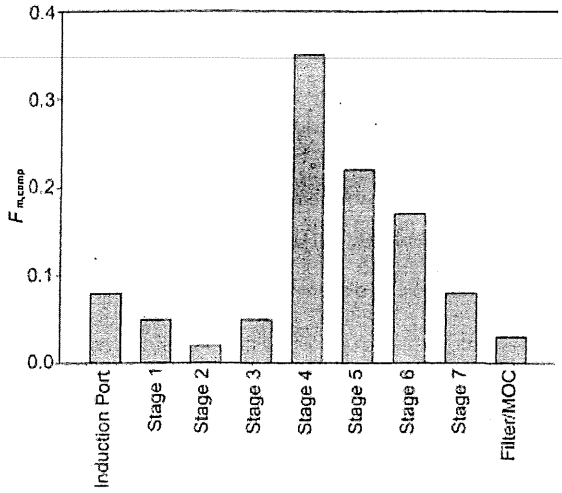

Figure 3. Example of Mass Fraction of Droplets Presented in Terms of Location within the Sampling System.

Determine the cumulative mass-weighted particle-size distribution of the aerosol size-fractionated by the impactor in accordance with the procedure given in ⟨601⟩. Starting at the filter, derive a cumulative mass versus effective cut-off diameter of the respective stages (see *Table 2* for the appropriate cut-off diameters at 15 L/min). Plot the cumulative fraction of drug substance versus cut-off diameter in a suitable format, e.g, logarithmic or log-probability format. Where appropriate, use this plot to determine by interpolation the fraction either less than a given size or between an upper and lower size limit.

Table 2. Cut-off Sizes for Apparatus 5 at 15 L/min

Stage	Cut-off Diameter (µm)
1	14.1
2	8.61
3	5.39
4	3.30
5	2.08
6	1.36
7	0.98

If necessary, and as appropriate, use this plot to determine values for the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD).

⟨1602⟩ SPACERS AND VALVED HOLDING CHAMBERS USED WITH INHALATION AEROSOLS—CHARACTERIZATION TESTS

1. INTRODUCTION
- 1.1 Background
- 1.2 Purpose
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4. MASS OF DRUG DELIVERED FROM A SPACER/VHC WHILE SIMULATING PATIENT TIDAL BREATHING

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4.1 Without Facemask
4.2 With Facemask

Change to read:

1. INTRODUCTION

1.1 Background

Spacers and valved holding chambers (VHCs) are widely used in conjunction with inhalation aerosols [commonly known as pressurized metered-dose inhalers (MDIs)]. When used correctly, VHCs assist with the administration of inhalation aerosols to patients who have poor coordination of MDI actuation and inhalation, and VHCs reduce oro-pharyngeal deposition of the drug. Patients using these spacers/VHCs in general breathe tidally when inhaling their medication, regardless of any delay, and this is particularly true for young and elderly users. Also, spacers and VHCs often come with a facemask instead of the mouthpiece normally supplied for inhalation aerosols. These add-on devices (spacers/VHCs) interface with the actuator/mouthpiece of the MDI and provide additional volume for the aerosol plume to develop. In this chapter, these aspects are addressed by providing patient-use appropriate scenarios that have been evaluated extensively.

Although existing methods for evaluating inhalation aerosols rely on sampling at a constant flow rate, spacers and VHCs may be used by patients who have poor inhalation technique (i.e., they delay inhalation after actuating the MDI). This chapter describes tests that are intended to provide information on how the spacer/VHC modifies the aerosol emitted by the MDI.

1.2 Purpose

The purpose of this chapter is to define potential standardized methods for characterizing the in vitro performance of a given MDI drug product with a specific spacer and VHC. It is recognized that spacer/VHC configurations will deliver a modified particle-size distribution and dose compared with the dose indicated on the label by the manufacturer of the MDI product, chiefly by collecting almost all of the portion of the dose contained in particles too large to be available for inhalation by the patient and potentially reach the lungs. This chapter does not advise on the outcome of such changes, rather the interpretation of data obtained using the methods described should be developed by the user after discussion with the appropriate receiving organization.

1.3 Rationale

This chapter includes separate testing by a multi-stage cascade impactor to determine aerosol aerodynamic particle size distribution (APSD) and tests mimicking fully coordinated and fully uncoordinated use, with tidal breathing simulated to establish emitted mass (EM) with the MDI-spacer/VHC coupled to a breathing simulator. ▲▲(USP 1-Dec-2019) It is now possible to combine both APSD and EM measurements by means of a mixing inlet. ▲▲(USP 1-Dec-2019) However, the more conservative approach adopted herein reflects the absence of method validation data for multiple marketed drug products delivered by MDI. Spacers and VHCs modify the APSD substantially from what is emitted by the MDI when used alone. In particular, almost all of the mass fraction of drug associated with particles having ballistic trajectories, by virtue of propellant expansion upon actuation, is contained within the add-on device. The user should refer to the Canadian Group standard for further interpretation of changes in APSD brought about by the presence of a spacer or VHC.

1.4 Recommendations

The use of the induction port identified in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ is recommended for compliance with the pharmacopeial standard. However, it is acknowledged that alternative designs of the induction port, as yet to be included as pharmacopeial standards, offer unique testing opportunities. These are either anatomically correct or idealized to have aerosol transport properties similar to an anatomically correct induction port. Given the widespread use of spacers/VHCs for infants and small children, it is important to note that many of these anatomically appropriate induction ports are scaled in terms of such potential users (e.g., the "Alberta" idealized throats, ▲▲(USP 1-Dec-2019) developed at the University of Edmonton, Canada). The user is free to adopt such an induction port but should specify the induction port design and age group with which the induction port will be used.

The evaluation of facemask performance requires a model face of the appropriate age range specified in the labeling for the add-on device (i.e., infant, small child, or adult). This arrangement is necessary because there is no other way to accurately simulate the magnitude of the dead space or test for the possibility of leak pathways between facemask and face, both of which are known to influence efficiency of the drug delivery. Some face models are available commercially. ▲▲(USP 1-Dec-2019) Therefore, because the use of a face model is an appropriate way to test these add-on devices, the user will need to either acquire a model from a source where age-appropriate face models have been developed and validated, or develop and validate his or her own design. Whichever pathway is chosen, a description of the model, in particular the dimensions of the face where the facemask comes into contact, should be provided in the data report to the recipient.

▲▲(USP 1-Dec-2019)

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1.5 Definitions of Key Terms Relating to This Chapter

Spacers: Open tubes that do not have a valve at the exit nearest the patient interface (either the mouthpiece or facemask) to retain the aerosol before the user can inhale (see *Figure 1*). Spacers simply increase the distance between the MDI mouthpiece and the mouth of the patient. If the patient exhales instead of inhaling, the medication will be blown out of the spacer and lost.

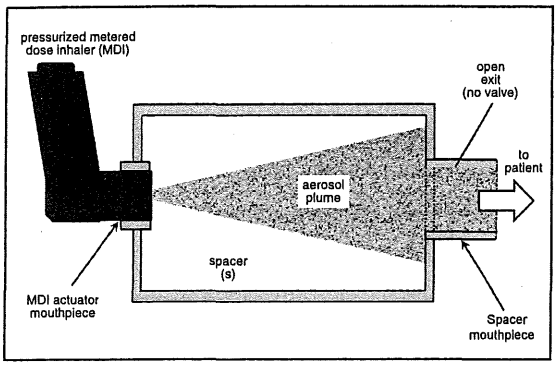

Figure 1. Open-tube spacer with no mechanism for aerosol conservation during exhalation.

VHCs: Contain at least one valve that opens to allow the patient to inhale aerosol on inspiration. This inhalation valve remains closed at other times during each breathing cycle (see *Figure 2*). Some VHCs may also contain an exhalation valve that is open only during exhalation to direct the exhaled flow away from the inhalation valve.

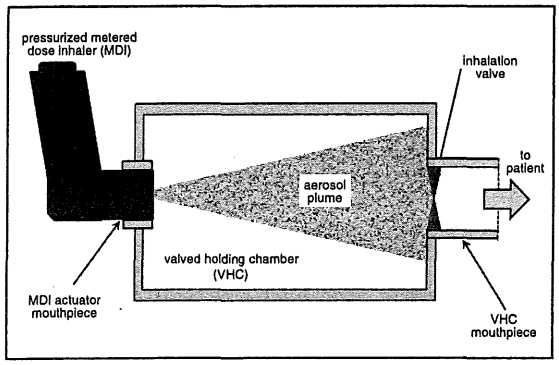

Figure 2. Conventional VHC with valve opening upon inhalation, accepting MDI with forward-firing actuator mouthpiece.

Integral actuator: A companion piece with some spacers and VHCs that accepts a MDI canister removed from its actuator-mouthpiece. A "reverse firing" feature may enable the design to be modified such that the built-in actuator faces away from rather than toward the user. In the example illustrated in *Figure 3*, the spacer operates in this way. It has some of the characteristics of a VHC, in that an air dam is created by closure of the valve located distally from the user on exhalation into the chamber. This type of spacer can therefore be evaluated as if it were a VHC, because the aerosol released on MDI actuation is conserved during exhalation.

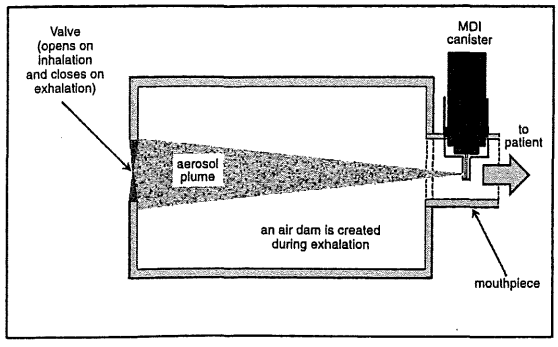

Figure 3. Holding chamber with reverse-firing integral actuator for MDI canister.

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1.6 Choice of Drug Product with which to Test

It is understood that testing will take place with each single active component within the drug product or with each strength of multi-strength drug products as required by the recipient of the data.

Change to read:

2. TEST METHOD SELECTION

2.1 Spacer/VHC Configurations

Four spacer/VHC configurations have been identified that require different test methods to complete the evaluation process (see *Table 1*).

Table 1. Identification of Test Configurations of the Spacer/VHC

Configuration	Spacer	VHC	Mouthpiece	Facemask
A	+[a]	–[b]	+	–
B	+	–	–	+
C	–	+	+	–
D	–	+	–	+

[a] + denotes that this item was part of the condition.
[b] – denotes that this item was not part of the condition.

In *Configuration A*, the device being tested is a spacer with a mouthpiece. Likewise, in *Configuration B*, the device under test is a spacer with a facemask. In *Configuration C*, the device is a VHC with a mouthpiece, and in *Configuration D* the device is a VHC with a facemask. The evaluation of devices without patient interface (i.e., those intended for use by patients on mechanical ventilation) is outside the scope of this chapter.

TESTS FOR CONFIGURATION A

Configuration A, spacer with mouthpiece (see *Figure 4*), comprises two tests: 1) APSD measurement with no delay (see *Part 1A*); and 2) EM by breathing simulation—fully coordinated (see *Part 2A*). Testing with a delay would be inappropriate for this configuration because the medication cannot be retained within the spacer because it has no valving mechanism.

TESTS FOR CONFIGURATION B

Configuration B, spacer with facemask (see *Figure 4*), comprises one test with the facemask in place: 1) EM by breathing simulation—fully coordinated (see *Part 3A*). ▲ [NOTE—The facemask should NOT be removed for this test, because it is important to be able to evaluate the effect of dead space between facemask and face, as well as the correct function of an exhalation valve, if provided.]▲ (USP 1-Dec-2019) It is recommended that this class of device be evaluated for APSD measurement with no delay (see *Part 1A*) by removing the facemask and coupling the spacer on-axis to the induction port entry with a suitable connector. Testing with delay would be inappropriate for this configuration, because the medication cannot be retained within the spacer because it has no valving mechanism. ▲ [NOTE—Removal of the facemask is acceptable for APSD determination, because the entire bolus of medication leaving the spacer is sampled in the continuous vacuum that is required to operate the cascade impactor.]▲ (USP 1-Dec-2019)

TESTS FOR CONFIGURATION C

Configuration C, VHC with mouthpiece (see *Figure 4*), comprises four tests: 1) APSD measurement with no delay (see *Part 1A*); 2) APSD measurement with delay (see *Part 1B*); 3) EM by breathing simulation—fully coordinated (see *Part 2A*); and 4) EM by breathing simulation—fully uncoordinated (see *Part 2B*).

TESTS FOR CONFIGURATION D

Configuration D, VHC with facemask (see *Figure 4*), comprises two tests with the facemask in place: 1) EM by breathing simulation—fully coordinated (see *Part 3A*); and 2) EM by breathing simulation—fully uncoordinated (see *Part 3B*). ▲ [NOTE—The facemask should NOT be removed for this test, because it is important to be able to evaluate the effect of dead space between facemask and face, as well as the correct function of an exhalation valve, if provided.]▲ (USP 1-Dec-2019) It is recommended that this class of device be evaluated for APSD measurement with and without delay (see *Parts 1A* and *1B*) by removing the facemask and coupling the VHC on-axis to the induction port entry with a suitable connector. ▲ [NOTE—Removal of the facemask is acceptable for APSD determination, because the entire bolus of medication leaving the spacer is sampled in the continuous vacuum that is required to operate the cascade impactor.]▲ (USP 1-Dec-2019)

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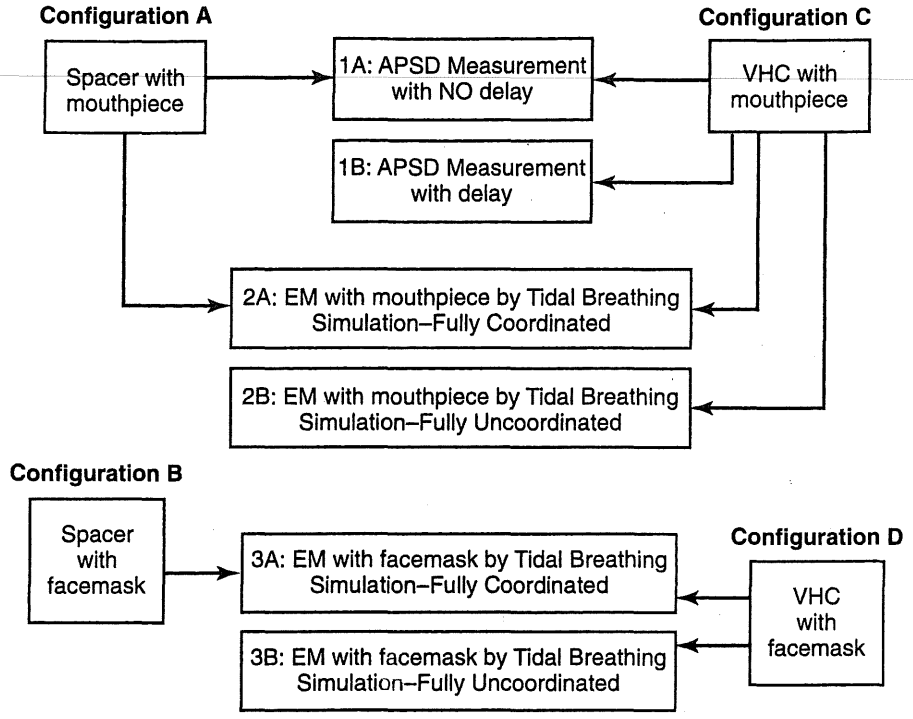

Figure 4. Decision tree for test selection by add-on device type.

2.2 Comments on Test Methods

The tests in *Parts 1A* and *1B* (see *Table 2*) are an extension of the procedures described in ⟨601⟩. However, sampling at a constant flow rate via a cascade impactor does not do more than enable the assessment of the APSD of the emitted aerosol. In the later parts of *Products for Nebulization—Characterization Tests* ⟨1601⟩, tests are described in which tidal breathing is simulated to assess spacer/VHC performance in terms of the delivered dose (equivalent to EM) in situations that are more representative of patient use. In *Part 1A* (see *Table 2*), the purpose is to enable the determination of metrics for which a direct comparison can be made with and without the add-on device. This methodology is the same as that described using either ▲the Andersen Cascade Impactor without pre-separator or the Next Generation Impactor without pre-separator (see ⟨601⟩)▲ (USP 1-Dec-2019) and is therefore not repeated here. In *Part 1B* (see *Table 2*), delayed sampling is introduced because add-on devices, especially VHCs, are widely prescribed for individuals who, for whatever reason, are unable to coordinate MDI actuation with the onset of inhalation as described for the MDI alone in "patient information" and "instructions for use". All of the tests in *Part 1* are undertaken with the facemask removed from the spacer/VHC equipped with this type of patient interface. This modification is allowed so that the device can be readily interfaced by fitting the facemask adapter directly to the entry of the induction port without the complication of a direct facemask-to-induction port connection, where internal dead space would likely be both ill-defined and unrepresentative of the "in-use" condition.

Table 2. In Vitro Tests for Spacers and VHCs

Test	Delay at Constant Flow Rate	Breathing Simulation	Suitability	Relevance to Patient Use Scenarios
Part 1A: Measurement of APSD with 4-L sample volume	None	Not applicable	Spacers and VHCs	Baseline performance compared with MDI alone
Part 1B: Measurement of APSD with 4-L sample volume	For example, 2, 5, or 10 s[a]	Not applicable	VHCs only	Simulation of a delay by a poorly coordinated user
Part 2▲A:▲ (USP 1-Dec-2019) Measurement of EM with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b]	Not applicable	Filter collection of aerosol simulating tidal breathing in coordinated ▲▲ (USP 1-Dec-2019) use	Spacers and VHCs	Simulation of optimum use (fully coordinated) with ▲pMDI▲ (USP 1-Dec-2019) actuation ▲▲ (USP 1-Dec-2019)

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Table 2. In Vitro Tests for Spacers and VHCs *(continued)*

Test	Delay at Constant Flow Rate	Breathing Simulation	Suitability	Relevance to Patient Use Scenarios
▲Part 2B: Measurement of EM with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b]	Not applicable	Filter collection of aerosol simulating tidal breathing in uncoordinated use	VHCs only	Simulation of fully uncoordinated use with pMDI actuation▲ (USP 1-Dec-2019)
Part 3▲A:▲ (USP 1-Dec-2019) Measurement of EM delivered by facemask with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b]	Not applicable	Filter collection of aerosol behind lips or at nares of model, simulating tidal breathing in coordinated ▲▲ (USP 1-Dec-2019) use	Spacers and VHCs	Simulation of optimum use (fully coordinated) with ▲pMDI▲ (USP 1-Dec-2019) actuation. ▲▲ (USP 1-Dec-2019) Simulation of use with facemask applied with a 1.6-kg force[c]
▲Part 3B: Measurement of EM delivered by facemask with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b]	Not applicable	Filter collection of aerosol behind lips or at nares of model, simulating tidal breathing in uncoordinated use	VHCs only	Simulation of fully uncoordinated use with pMDI actuation. Simulation of use with facemask applied with a 1.6-kg force[c]▲ (USP 1-Dec-2019)

[a] Other delay intervals may also be used as required, if justified.
[b] Representative breathing patterns for different patient ages are listed in *Table 3*; other patterns may be used as required, if justified.
[c] 1.6-kg applied force has been shown to be clinically appropriate, but the force used may differ from this value if justified.

The tests in *Parts 2* and *3*, depending on whether a mouthpiece or facemask is present as the patient interface, respectively (see *Table 2*), evaluate the performance of the inhalation valve (and exhalation valve, if equipped) of the add-on device. This evaluation is accomplished by simulating tidal breathing, with parameters that are appropriate for the intended user group (e.g., infant, small child, or adult). This testing approach creates the continually varying flow conditions that are expected in use. Note that in the determination of EM, a sufficient number of inhalations should be taken in accordance with the instructions for use of the VHC, to ensure that the intended amount of drug is delivered.

Spacers/VHCs using a facemask as the patient interface should be evaluated ideally with the facemask in place (see *1.3 Rationale*). The desirable approach is such that in vitro testing makes use of a model face that is representative of the age range for which the spacer/VHC is intended. There are currently limited commercially available model faces; however, the peer-reviewed literature contains several articles providing the necessary technical information to enable suitable age-appropriate model(s) to be created for the purpose of testing spacer/VHCs. It is also important that the surfaces of the face coming into contact with the facemask have mechanical characteristics (e.g., deformability) that are representative of the skin and underlying soft tissues when the facemask is applied to the face with an appropriate force. In *Part 3* (see *Table 2*), the spacer/VHC is evaluated by a breathing simulation with the facemask fitted, mimicking similar fully coordinated use as defined in *Part 2A* and also fully uncoordinated use as defined in *Part 2B* (see *Table 2*). By comparing the EM of the drug with and without the use of a facemask, it is possible to assess the influence of the facemask.

[NOTE—Not all of the tests described in this chapter are applicable to open-tube spacers because such devices require fully coordinated use; otherwise, drug delivery will be significantly reduced. *Figure 4* shows the decision tree to be followed so that the tests chosen are appropriate to the type of add-on device being evaluated.]

Table 2 further defines the test, method, applicable device, and relevance to patient use scenarios of the procedures that are described in this chapter. Example delay intervals of 2, 5, and 10 s are noted in the table, although other delay intervals may be substituted or added as required and justified. For the testing in *Parts 3*▲*A* and *B*▲ (USP 1-Dec-2019) (see *Figure 4*), it is recognized that currently there are limited commercially available face models representing the most widely recognized age categories, which may be required (i.e., infant, small child, and adult). The user is advised to select an appropriate commercial model or develop his or her own, providing justification for the selection as required. Additionally, *Table 3* shows representative tidal-breathing patterns for the various patient age categories from neonate to adult.

Table 3. Representative Tidal Breathing Patterns[a]

Parameter	Pediatric			Adult	
	Neonate	Infant	Small Child	Normal 1[b]	Normal 2[b]
Tidal volume (mL)	25	50	155	770	500
Frequency (min^{-1})	40	30	25	12	13
I/E ratio[c]	1:3	1:3	1:2	1:2	1:2

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Table 3. Representative Tidal Breathing Patterns[a] *(continued)*

Parameter	Pediatric			Adult	
	Neonate	Infant	Small Child	Normal 1[b]	Normal 2[b]
Minute volume (mL)	1000	1500	3875	9240	6500

[a] With the permission of the Canadian Standards Association (operating as the CSA Group), material is reproduced from CSA Group standard CAN/CSA-Z264.1-02 (R2011), "Spacers and Holding Chambers for Use with Metered-Dose Inhalers", which is copyrighted by CSA Group, 5060 Spectrum Way, Suite 100, Mississauga ON, L4W 5N6. This material is not the complete and official position of the CSA Group on the referenced subject, which is represented solely by the standard in its entirety. Although use of the material has been authorized, CSA is not responsible for the manner in which the data are presented, nor for any interpretations thereof. For more information or to purchase standards from the CSA Group, please visit shop.csa.ca/ or call 1-800-463-6727.

[b] *Normal 1* represents a resting adult of large build; *Normal 2* represents an active adult of normal build.

[c] *I/E ratio* = inspiratory time/expiratory time.

▲Testing is undertaken (see *3.1 No Delay between the MDI Actuation and Sampling Onset*) to ensure that there are no leakages of ambient air into the measurement apparatus. The purpose is not to evaluate leakages associated with the spacer/VHC. Such testing would be impractical because of the variety of adapters that would be needed to interface the flow meter with the different designs associated with these add-on devices.▲ (USP 1-Dec-2019)

Change to read:

3. MEASUREMENT OF APSD

3.1 No Delay between the MDI Actuation and Sampling Onset

BACKGROUND

In *Part 1A* (see *Figure 4*), the measurement of APSD from the spacer or VHC with no delay following actuation of the inhaler is defined as an important test of the optimum performance of the device. This test also provides information with which to compare the in vitro performance of the MDI with and without the add-on device present.

TEST PROTOCOLS

If the spacer/VHC is intended for adult use, and comparison is being made with the MDI alone, follow ▲one of▲ (USP 1-Dec-2019) the procedures given for ▲inhalation aerosols and sprays,▲ (USP 1-Dec-2019) as required. Alternatively, ▲the pediatric version of▲ (USP 1-Dec-2019) the Marple-Miller Cascade Impactor ▲(Model 150P)▲ (USP 1-Dec-2019) may be used.

If the spacer/VHC is intended for neonates, infants, or small children, it may be appropriate to use an alternative apparatus that operates at the reduced flow rate(s) more appropriate for these classes of patients. Examples are ▲the Next Generation Impactor (without pre-separator)▲ (USP 1-Dec-2019) operated at 15.0 L/min or the pediatric version of ▲▲ (USP 1-Dec-2019) the Marple-Miller Cascade Impactor (Model 150P) that functions at either 4.9 or 12.0 L/min.

▲Set up the cascade impactor as described in the manufacturer's literature. To ensure efficient particle capture, coat appropriately the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid unless this has been demonstrated to be unnecessary. Connect an induction port with internal dimensions as defined in ⟨601⟩ to the impactor inlet. Connect the impactor to a vacuum pump via a suitable flow control valve.

Attach a flowmeter capable of providing volumetric flow rate, within the range of operation leaving the meter, to the induction port, ensuring an airtight seal. Verify that the flow rate for the test is within ±5% of the specified flow rate. This is achieved by applying a vacuum to the test apparatus by means of the flow control valve located between the cascade impactor and vacuum source. Turn off the source of vacuum to this apparatus after this check has been completed and remove the flow meter.

Attach a suitable coupling for the spacer/VHC to the induction port.▲ (USP 1-Dec-2019) Conduct the ▲remainder of the▲ (USP 1-Dec-2019) evaluation with the mouthpiece of the spacer or VHC, if so equipped, connected to the test apparatus. If the spacer or VHC is equipped with a facemask, remove the facemask and perform the evaluation with the add-on device connected to the test apparatus by means of the facemask adapter.

▲Prepare the spacer/VHC▲ (USP 1-Dec-2019) for the test by washing in accordance with the manufacturer's instructions, if so indicated. If no instructions for preparation are provided, test the device out of the package without prewashing, and note this information in the test report.

Connect the spacer or VHC via the mouthpiece or facemask adapter to the entry to the induction port described in ⟨601⟩. This connection can be made either with a short piece of flexible tubing or with a purpose-built coupling that ensures on-axis alignment with the entry to the induction port. If flexible tubing is used for this connection, ensure that the add-on device is supported in such a way that its long axis aligns with the axis of the induction port entry. Care should be taken to ensure that the coupling, however constructed, does not result in a significant loss of the drug and/or change in measured APSD.

▲▲ (USP 1-Dec-2019)

The practice of actuating the MDI with the spacer or VHC initially disconnected from the induction port and then rapidly connecting the add-on device after MDI actuation is not recommended, because there is always a small delay interval and medication will be lost in an uncontrolled manner from an open-tube spacer if this procedure is followed.

Follow the directions in the patient information and instructions for use for the MDI to prime it before inserting into the MDI adapter of the spacer/VHC. In the case of an add-on device equipped with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter and clean the external valve stem with a suitable

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wipe. Insert the canister valve stem into the receptacle following the instructions for use for the particular add-on device, taking care to avoid premature actuation.

In cases where more than one actuation of the MDI is needed to collect sufficient mass of drug product, follow the directions in the patient information and instructions for use; if no directions are provided, allow a minimum of 30 s between actuations. Turn on the vacuum to the cascade impactor before delivering any actuations. It is important to deliver the minimum number of actuations to obtain an adequate sample of the size-fractionated drug mass for measurements to be made to the same degree of precision as would be the case if the MDI was evaluated alone.

After the MDI actuation and aerosol sampling are completed, recover the drug from each component of the cascade impactor, including the induction port, and also from within the spacer/VHC by using a validated method appropriate to the product being measured. Carefully remove the backup filter of the cascade impactor, whose purpose is to capture the drug mass passing the last impaction stage, from its location and place it in a suitable container. For each sample, add a predetermined volume of a suitable solvent to the container and agitate to dissolve the collected drug. Transfer a sample to a cuvette or vial for drug assay using a syringe equipped with an in-line filter to retain any suspended material. Care must be taken with the recovery of a drug retained by the add-on device, because a greater volume of solvent may be needed, compared with volumes needed for recovery from each impaction plate/cup of the impactor. Perform separate assays to measure the mass of drug recovered from each component, using a validated procedure.

Repeat the measurement with the required number of devices and replicates per device. Typically, five separate spacers or VHCs are each tested once, but other testing protocols may be adopted as required, if justified. These protocols may include replicate measurements per device to obtain information about intra- as well as inter-device performance.

MEASUREMENTS

Total mass of drug collected: This procedure is not a test of the product or add-on device but serves as a system suitability test to ensure that the results are valid. The total mass of drug collected comprises the sum of the drug mass in all of the components, including the MDI mouthpiece and from within the spacer/VHC [material balance (MB)] divided by the number of actuations of the MDI. Note that typically up to 5% of the target-delivered label claim may be retained by the cascade impaction apparatus as inter-stage wall losses.

Total mass of drug emitted: The total mass of drug emitted from the spacer/VHC with zero seconds delay (delivered dose, equivalent to EM) is determined from the sum of the values for the mass of drug collected in all of the components of the test apparatus divided by the number of actuations of the MDI. Spacers and VHCs are intended to reduce the mass of coarse particles inhaled by the patient; therefore, an important part of data interpretation of the APSD should involve separate assessments of the pertinent subfractions relevant to the inhalation aerosol product. The precise size limits for these subfractions will agree with the recipient of the test data. However, to provide initial guidance on this matter, it is suggested that the following be considered as a minimum:

- Determination of the mass of drug retained by the spacer/VHC together with the mass recovered from the induction port
- Stage-by-stage profile of the remaining EM

Further interpretation of these data is at the discretion of the user. The performance of the spacer/VHC is compared with that of the MDI alone, following the procedure described in ⟨601⟩.

3.2 Delay between the MDI Actuation and Sampling Onset

[NOTE—This test is inappropriate for spacers.]

BACKGROUND

This test requires the adaptation of ▲the Andersen Cascade Impactor without pre-separator and the Next Generation Impactor without pre-separator (see ⟨601⟩)▲ (USP 1-Dec-2019) to accept a means by which the delay interval can be realized. The actuation of the MDI into the VHC on test with its mouthpiece or facemask adapter disconnected from the entry to the induction port, followed by connection to the port with the vacuum applied to the apparatus immediately after the delay interval has elapsed, is difficult to perform accurately and may result in leakage or an inaccurate realization of the delay interval. For these reasons, an apparatus of the type described in *Figure 5* is recommended, because it enables the VHC mouthpiece/facemask adapter to remain in position throughout the measurement. The methodology for delay testing is based on the apparatus illustrated, but other equipment offering similar capability may be used if justified.

TEST PROTOCOLS

The vertically mounted shutter plate comprises a circular opening in its upper half and is mounted at setup such that the gap between the VHC mouthpiece/facemask adapter and induction port entry is closed, as illustrated (see *Figure 5*). The induction port and VHC adapter fit tightly into the mounting block. In this position, when vacuum is applied to the test apparatus, air is sampled by the apparatus at the desired flow rate via a bypass channel on the side of the adapter facing the induction port. This arrangement avoids the need to start the flow through the apparatus after the delay interval has elapsed, so that the cascade impactor is always operated at a constant flow rate.

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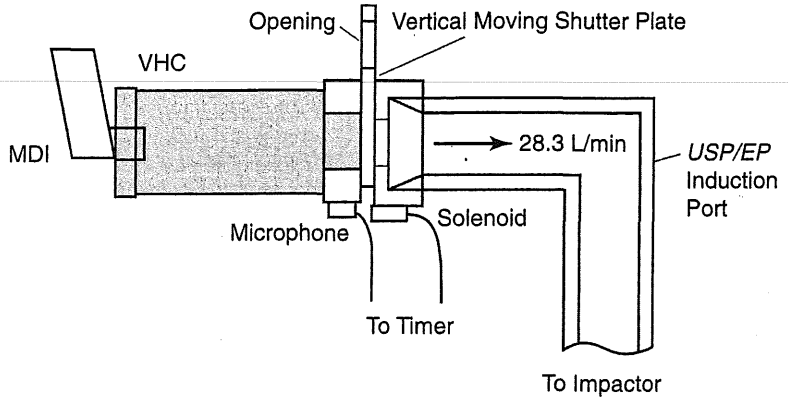

Figure 5. A configuration for an apparatus to assess the effect of a delay between the MDI actuation and sampling onset.

In the configuration described in *Figure 5*, when the MDI is actuated, a microphone located on the adapter block detects the sound emitted at actuation of the MDI, starting a timer that operates a solenoid valve that retracts a pin immediately after the preset delay interval has expired. This process permits gravity to operate on the shutter, which drops to the lower position in which the aperture is aligned with the VHC mouthpiece/facemask adapter and induction port entrance. The aerosol retained within the VHC is sampled as soon as the shutter moves to the "open" position. This procedure avoids the risk of capturing any "blow-by" aerosol that might escape the VHC as the propellant expands immediately following MDI actuation but would not be inhaled by a user. If blow-by is observed, it should be noted in the test report. In the configuration shown in *Figure 5*, the adapter introduces <5 mL of additional volume to the aerosol pathway from the VHC to the filter, and the minimum delay interval achievable is 1 s.

Example delay intervals of 2, 5, and 10 s have been noted earlier in this chapter, although other delay interval(s) may be substituted or added if required and justified.

▲Connect the impactor to a vacuum pump via a suitable flow control valve.

The delay apparatus, if constructed in accordance with *Figure 5* will introduce leakage into the apparatus, once attached to the induction port because the bypass flow passage is open in order that in-flow comes in via this passage rather than through the VHC before the shutter drops after the prescribed delay period. The flow rate verification test is therefore conducted before the delay apparatus is attached. Connect an induction port with internal dimensions as defined in ⟨601⟩ to the impactor inlet. Attach a flowmeter capable of providing volumetric flow rate, within the range of operation leaving the meter, to the coupling, ensuring an airtight seal. Verify that the flow rate for the test is within ±5% of the specified flow rate. This is achieved by applying a vacuum to the test apparatus by means of the flow control valve located between the cascade impactor and vacuum source. Turn off the source of vacuum to this apparatus after this check has been completed and remove the flow meter.▲ (USP 1-Dec-2019)

Securely attach the exit port from the delay apparatus to the entry of the induction port. ▲Conduct the remainder of the evaluation with the mouthpiece of the spacer or VHC, if so equipped, connected to the test apparatus. If the spacer or VHC is equipped with a facemask, remove the facemask and perform the evaluation with the add-on device connected to the test apparatus by means of the facemask adapter. If this is the first testing of the spacer/VHC or the intention is to evaluate the add-on as new for each delay condition, prepare it for the test by washing in accordance with the manufacturer's instructions, if so indicated. If no instructions for preparation are provided, test the device out of the package without prewashing, and note this information in the test report.▲ (USP 1-Dec-2019) Connect the VHC via the patient interface (mouthpiece or facemask adapter) to the entry port of the delay apparatus. ▲▲ (USP 1-Dec-2019)

Before actuating the MDI for the first time into the VHC being tested, ensure that the delay apparatus is set such that the shutter is in the "up" or "closed" position. Select the desired delay interval for the timer.

Follow the directions in the patient information and instructions for use before inserting the actuator/mouthpiece of the MDI into the MDI adapter of the VHC before the first actuation into the test apparatus. If the spacer/VHC is designed with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle following the instructions for the particular add-on device, taking care to avoid premature actuation.

In cases where more than one actuation of the MDI is needed to collect sufficient mass of drug product, follow the directions in the patient instructions, or if no direction is provided, allow a minimum of 30 s between actuations. Deliver the minimum number of actuations needed to obtain an adequate sample of the size-fractionated drug mass so that measurements can be made to the same degree of precision as if the MDI was evaluated alone. Reposition the shutter of the delay apparatus in the up or closed position, and check that the timer is set for the desired delay after each MDI actuation. Maintain the vacuum through the entire sequence until all actuations are completed.

After the sampling part of the measurement is completed, recover the drug from each component of the cascade impactor, including the induction port, and from within the spacer/VHC by using a validated method appropriate to the specific product. In the configuration shown in *Figure 5*, the surface area of the shutter that is exposed to aerosol particles is minimal. Therefore, recovery of drug from this component needs to be done only for the most accurate work; in which case, the inner surfaces of the aperture in the shutter plate and the exit port are washed with recovery solvent. Care should be taken with the recovery of drug retained by the VHC, because a greater volume of solvent than is needed for recovery from each impaction plate/cup of the impactor may be needed to be sure that drug is quantitatively recovered. Assay for the mass of drug recovered from each component separately by using a validated procedure.

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Repeat the measurement with the required number of devices and replicates per device. Typically, five separate spacers or VHCs are each tested once, but other designs, including replicate measurements per device, may be adopted as required.

MEASUREMENTS

Total mass of drug collected: The total mass of drug collected in all of the components, including the MDI mouthpiece and from within the spacer/VHC (MB) is divided by the number of actuations of the MDI. The component of the dose that may escape past the inhalation valve of the VHC on MDI actuation (due to momentary pressurization of the interior of the chamber) is termed "blow-by", and it may not be captured for the assay. If this phenomenon is observed, it must be noted.

[NOTE—Quantification of blow-by is not a practical proposition because the act of collecting the aerosol will inevitably apply back-pressure to the valve, reducing or eliminating the phenomenon altogether.]

The performance of the spacer/VHC tested with delay (see *3.2 Delay between the MDI Actuation and Sampling Onset*) is compared with that of the spacer/VHC tested with no delay (see *3.1 No Delay between the MDI Actuation and Sampling Onset*).

VHCs are intended to reduce the mass of coarse particles inhaled by the patient, so an important part of data interpretation of the APSD should involve separate assessments of the pertinent subfractions relevant to the inhalation aerosol product. The precise size limits for these subfractions will agree with the recipient of the test data. However, to provide initial guidance on this matter, it is suggested that the following be considered as a minimum:

- Determination of the mass of drug retained by the spacer/VHC together with the mass recovered from the induction port
- Stage-by-stage profile of the remaining EM

Further interpretation of these data is at the discretion of the user.

Change to read:

4. MASS OF DRUG DELIVERED FROM A SPACER/VHC WHILE SIMULATING PATIENT TIDAL BREATHING

4.1 Without Facemask

Cascade impactors used in the assessment of APSD are designed to operate at fixed flow rates. However well the add-on device may perform during an in vitro test of function using such an apparatus, the operation of critical moving components (i.e., inhalation and exhalation valves of VHCs) is not evaluated in the way that these components would perform when the VHC is used by the patient. An additional test that simulates tidal breathing is therefore included, because this type of respiratory pattern is most commonly encountered with patients using an add-on device, particularly neonates, infants, and children who are incapable of a forced inhalation maneuver. Several representative, age-related breathing patterns are listed in *Table 3*.

There is no breathing simulator recognized in ⟨601⟩. However, ⟨1601⟩ provides for the use of a commercially available breathing simulator that is able to generate the same breathing profiles as those specified in *Table 3*. The methodology provided assumes that equipment meeting this standard is available. *Figure 6* illustrates schematically a setup that has been effective for these measurements, but other configurations may be used as required.

The filter system used to collect the aerosol at the mouthpiece/facemask adapter of the spacer/VHC should be a suitably validated, low-resistance filter capable of quantitatively collecting the aerosol and enabling recovery of the drug substance with use of an appropriate solvent. If the filter is contained in its own housing, the dead volume of the filter casing must not exceed 10% of the tidal volume used in the breath simulation. This restriction will likely make it necessary to use filters without a separate housing when performing tests that simulate neonatal or infant use.

The first part of the test is suitable for both spacers and VHCs, because it simulates the delivery of medication mimicking a fully coordinated user actuating the MDI at the onset of inhalation. However, the second part of the test is only suitable for VHCs because the MDI is actuated at the onset of exhalation to simulate a fully uncoordinated user.

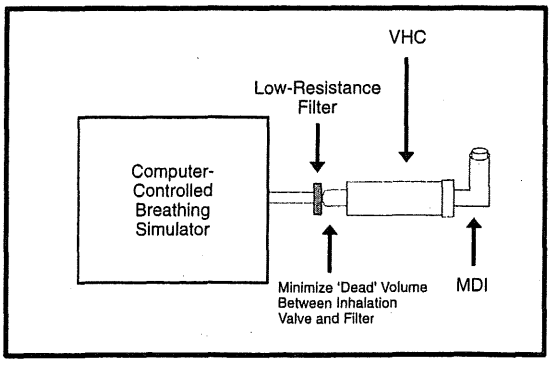

Figure 6. VHC evaluation by breathing simulator.

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TEST, PART 1

Set the breathing simulator to the required breathing pattern (identified from *Table 3*) in accordance with the manufacturer's instructions. Alternative breathing patterns may be used if required. Ensure that the simulator has been calibrated before use so that the actual volumes and frequency are within ±5% of the indicated value during each breathing cycle. Calibration syringes are available from various suppliers of mechanical ventilation equipment to verify volumes. To verify frequency, the method varies among breathing simulator manufacturers, and therefore the advice of the particular supplier/manufacturer should be sought if the method is not explicitly stated in the operating instructions for the apparatus.

Ensure that the selected flow waveform from the computer-controlled breathing simulator (*Figure 6*) is stable before proceeding. Prepare each device in the group of spacers or VHCs to be tested by washing in accordance with the manufacturer's instructions, if indicated. If no instructions for preparation are provided, test the devices out of package without prewashing and note this fact in the test report.

Connect the mouthpiece or facemask adapter of the spacer/VHC to the breathing simulator by means of a short length of flexible hose. Locate the aerosol filter as close as possible to the mouthpiece of the spacer/VHC using an adapter or other means that will enable a leak-tight seal to be formed with the minimum amount of space (dead volume) between the device and the filter. Ensure that the adapter does not restrict the path of the aerosol.

Filtrete is a suitable electret filter medium ▲▲(USP 1-Dec-2019) that readily releases collected drug quantitatively upon addition of a suitable solvent during the assay procedure. However, other suitable filter media may be used provided that the tester is satisfied that the filter is capable of collecting the entire emitted dose and that drug recovery for assay is quantitative.

Follow the directions in the patient information and instructions for use for the MDI to prime it before inserting into the MDI adapter of the spacer/VHC before the first actuation into the test apparatus. In the case of an add-on device equipped with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle by following the instructions for use for the particular add-on device, taking care to avoid premature actuation. This test typically should require only one actuation of the MDI into the spacer/VHC per determination. However, for certain highly potent products delivering low unit mass of drug per actuation, more than one actuation of the MDI may be needed to collect a sufficient mass of drug product. Under such circumstances, follow the directions in the patient information and instructions for use for the MDI, or allow a minimum of 30 s between actuations if no direction is provided. It is important to deliver the minimum number of actuations to obtain an adequate sample of the drug mass for measurements to be made with acceptable precision.

For the first part of the test, perform a single actuation timed to coincide with the *beginning* of an inhalation. Allow sampling to occur for five additional breathing cycles for the actuation. If additional actuations are required to improve analytical sensitivity, ensure that the sampling time is of sufficient length for the spacer/VHC to be emptied of remaining aerosol before the next actuation is delivered, and do not disconnect the spacer/VHC from the breathing simulator between actuations.

Remove the filter carefully from its location and place it in a suitable container. Add a predetermined volume of a suitable solvent to the container and agitate to dissolve the collected drug. Transfer a sample to a cuvette or vial for drug assay using a syringe equipped with an in-line filter to retain any suspended material. Recover the mass of drug from the filter by using a validated procedure appropriate to the specific product, and assay for the mass of drug collected by using a validated procedure. Calculate the total mass of drug per actuation; this is the emitted mass in the fully coordinated condition (EM_c). Repeat the measurement with the required number of devices and replicates per device.

TEST, PART 2

[NOTE—This part of the test is inappropriate for spacers.] For the second part of the test, either clean the VHC between measurements or evaluate a new VHC out of its packaging, as required and justified. Report whether cleaning or replacement of the VHC was carried out. Repeat the procedure above in *Test, Part 1* with the same VHC, this time actuating the MDI timed to coincide with the onset of exhalation. Calculate the total mass of drug per actuation; this is the EM in the fully uncoordinated condition (EM_{uc}). Repeat the measurement with the required number of devices and replicates per device.

Calculate the ratio of EM_c with the EM_{uc}.

4.2 With Facemask

TEST, PART 1

The purpose of this test is to compare the EM from a spacer/VHC equipped with a facemask to that obtained in the fully coordinated simulation with the facemask removed. Facemasks are widely prescribed for infants, small children, and adults who lack coordination to use a mouthpiece-based product. The evaluation of spacers/VHCs supplied with a facemask, rather than a mouthpiece, requires additional attention beyond the methods described previously, because the facemask itself has a major part to play in the transport of the drug-containing aerosol particles from the add-on device to the patient (*Figure 7*). It is therefore important that the facemask be tested in situ as part of the add-on device, rather than separately. A critical component of the test apparatus is the representation of a human face appropriate to the age range for which the add-on device is intended (e.g., neonate, infant, small child, or adult). There are few models that can be defined as an apparatus in a way that is similar to what is done for cascade impactors in the aerodynamic particle size analysis of MDI-generated aerosols, although some face models are currently available commercially. Ideally, standardized, age-appropriate models would be preferred. ▲▲(USP 1-Dec-2019)

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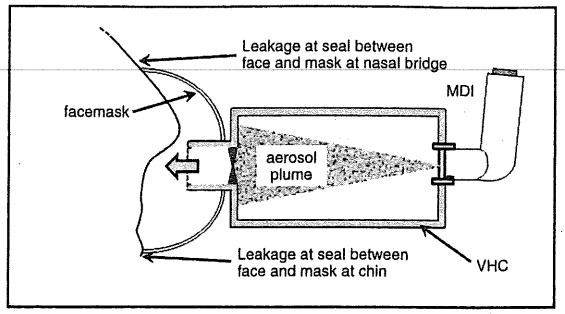

Figure 7. VHC with facemask applied to face.

Ideal attributes for face models are the following:

- Appropriate facial dimensions for the intended user age range
- Ability to apply the facemask with the predicted amount of dead space when it is applied with a clinically appropriate force to the model
- Physiologically accurate soft facial tissue modeling around the chin, cheeks, and nose where the facemask makes contact
- Means of correctly mounting the spacer/VHC so that the facemask is oriented with the correct alignment to the face, as would occur when in use by a patient

[NOTE—Some models may include anatomically accurate realization of the upper airway (naso- or oropharynx), with the aerosol collection filter located at the distal port of the model representing the entry to the lungs.]

For the sake of simplicity and to realize a measure of spacer/VHC performance comparable to the measure obtained with facemask removed in *Parts 2*▲*A* and *B*▲ (USP 1-Dec-2019) (see *Figure 4*), the default assumption is that the model face simulates open-mouth breathing without an anatomically accurate upper airway, and that the aerosol collection filter is located in a cavity immediately behind the lips of the model. Alternatively, if an obligate nasal breathing infant model is being used, then the filter can be placed behind the nares. The mass of drug is reported as the emitted mass ($EM_{facemask}$).

Select a face model appropriate to the intended age range for the spacer/VHC facemask, and mount the face model in an appropriate fixture. The fixture should enable the facemask to be located at an appropriate angle to the face model, such that an effective seal between the facemask and face model is created with a clinically appropriate force. This is typically a loading force of 1.6 kg but may differ from this value if justified by the design of the facemask. The arrangement shown schematically in *Figure 8* is one way of achieving the desired result, but other approaches may be adopted as required, if justified.

Locate and secure the filter media in the cavity behind the lips of the face model. Filtrete is a suitable electret filter medium ▲▲ (USP 1-Dec-2019) that readily releases collected drug quantitatively upon addition of a suitable solvent during the assay procedure. However, another suitable filter medium may be used, provided that the tester is satisfied that it is capable of collecting the entire emitted dose, and that drug recovery for the assay is quantitative.

Verify that a seal has been obtained between the facemask and face model. This is conveniently done by connecting the outlet (distal) port of the face model, using a short length of flexible hose, to an in-line calibrated gas flow meter whose distal port is in turn connected to the vacuum source via a regulating valve capable of setting the flow rate to a suitable value, typically 30 L/min. Connect a similar flow meter to the port for the MDI (MDI adapter) on the spacer/VHC, and ensure that the mask is positioned in order to maximize flow rate. ▲▲ (USP 1-Dec-2019)

[NOTE—Leakage from the facemask to face seal, as detected by lower flow rate, is known to reduce drug delivery.]

Turn off the vacuum source after this check has been completed, and disconnect the flexible hose from the in-line flow meter. Remove the upstream flow meter from the MDI adapter of the spacer/VHC.

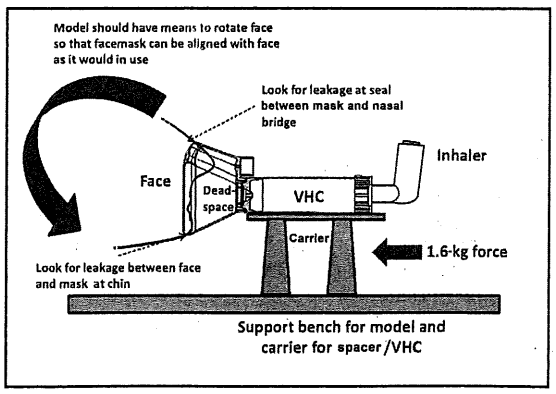

Figure 8. Mount for face model with alignment fixture including carriage for spacer/VHC; a fixed (i.e., 1.6-kg) weight acts on the pulley below the VHC and draws the carriage toward the face with the required force.

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Connect the outlet (distal) port of the face model to the appropriate port of the breathing simulator using a short length of flexible hose. Set the breathing simulator to the required breathing pattern (identified from *Table 3*) in accordance with the manufacturer's instructions. Alternative breathing patterns may be used if required. Ensure that the simulator has been calibrated before use so that the actual volumes and frequency are within ±5% of the indicated value during each breathing cycle. Calibration syringes are available from various suppliers of mechanical ventilation equipment to verify volumes. To verify frequency, the method varies among breathing simulator manufacturers, and therefore the advice of the particular supplier/manufacturer should be sought if the method is not explicitly stated in the operating instructions for the apparatus. Ensure that the selected flow waveform from the breathing simulator is stable before proceeding.

Follow the directions in the patient information and instructions for use for the MDI to prime it before inserting it into the MDI adapter of the spacer/VHC before the first actuation into the test apparatus. In the case of an add-on device equipped with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter, and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle according to the instructions for use for the particular add-on device, taking care to avoid premature actuation. Typically, it should require only one actuation of the MDI into the spacer/VHC per determination. However, for certain highly potent products delivering a low-unit mass of drug per actuation, more than one actuation of the MDI may be needed to collect a sufficient mass of drug product. Under such circumstances, follow the directions in the patient instructions or allow a minimum of 30 s between actuations if no direction is provided. Deliver the minimum number of actuations needed to obtain an adequate sample of the drug so that mass measurements can be made with acceptable precision.

Perform a single actuation in the spacer/VHC, timed to coincide with the beginning of an inhalation (see *Figure 4*, *Part 3A*). Allow sampling to occur for five additional breathing cycles for the actuation. If additional actuations are required to improve analytical sensitivity, ensure that the sampling time is of sufficient duration for the spacer/VHC to be emptied of remaining aerosol before the next actuation is delivered, and do not disconnect the spacer/VHC from the breathing simulator between actuations.

Following the tests, remove the filter carefully from its location and place it in a suitable container. Add a predetermined volume of a suitable solvent to the container and agitate to dissolve the collected drug. Transfer a sample to a cuvette or vial for drug assay using a syringe equipped with an in-line filter to retain any suspended material. Recover the mass of drug from the filter by using a validated method appropriate to the specific product, and assay for the mass of drug collected by using a validated procedure. Calculate the total mass of drug per actuation; this is the emitted mass in the fully coordinated condition with facemask fitted to the spacer/VHC (EM_{c-fm}). Repeat the measurement with the required number of devices and replicates per device.

The values of EM_c from the test undertaken in *Part 2* ▲*A*▲ (USP 1-Dec-2019) (see *Figure 4*) in which the facemask adapter was removed for comparison of EM, simulating fully coordinated use by a breathing simulator can be compared to the EM_{c-fm} in order to understand the impact of the facemask on drug delivery.

TEST, PART 2

For a VHC only, the same process can be repeated, but with the MDI actuation(s) timed to coincide with the beginning of exhalation (rather than inhalation) (see *Figure 4*, *Part 3B*). In this instance, the mass of drug collected per actuation is the emitted mass in the uncoordinated condition (EM_{uc-fm}). The ratio of EM_{c-fm} from *Part 1* ▲of this test▲ (USP 1-Dec-2019) and the EM_{uc-fm} can now be calculated.

⟨1644⟩ THEORY AND PRACTICE OF ELECTRICAL CONDUCTIVITY MEASUREMENTS OF SOLUTIONS

This general chapter provides information in support of instrumental methods for procedures that measure electrical conductivity. Pharmaceutical applications include: chemical dosing, cleaning in place, fermentation control, and liquid mixing verification, among others. Although the general chapter focuses on aqueous systems, conductivity measurements can be extended to organic fluids. The general chapter also focuses on contacting conductivity measurements and does not cover applications which may use noncontacting inductive conductivity. After an introduction, the general chapter covers the following major topics: theory of operation, operational considerations, calibration, and operation for at-line, in-line, and off-line measurement procedures.

INTRODUCTION

Conductivity is the measurement of the ability of a fluid to conduct electricity via its chemical ions. The ability of any ion to electrically conduct is directly related to its ion mobility. Some of the common applications of conductivity measurements include water treatment and purification, clean-in-place process fluid management, fermentation process monitoring, dosing applications, nutrient media preparation, buffer production (e.g., distribution and dilution for dialysis and chromatography applications), chromatography detection of gradient and eluent, active pharmaceutical ingredient chemical synthesis, and concentration determination of basic chemicals. Fluids should be measured in a single homogenous phase—i.e., conductivity should not be applied to mixed immiscible fluids unless they are separated. Electrical conductivity measurements cannot be applied to solids or gases, but they can be applied to the condensate of gases.

Besides its use to monitor ionic concentrations of process fluids, conductivity is also useful for the detection of ionic impurities in compendial waters (see *Water Conductivity* ⟨645⟩) and for the detection of ionic impurities in organic matrices.

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The measurement is non–ion-specific, and all ions respond with different efficiency or equivalent conductance, λ. Despite the lack of ionic specificity, conductivity is a valuable laboratory and process tool for measurement and control of total ionic content because it is proportional to the sum of the concentration of each ionic species (anions and cations) as described in *Equation 1*:

$$\kappa = 1000 \sum_{i}^{all\ ions} C_i \lambda_i$$

where κ is the conductivity (S/cm), C_i is the concentration of chemical ion *i* (mole/L), and λ_i is the specific conductance of ion *i* (S · cm²/mole). Though S/m is the appropriate SI unit for conductivity (i.e., the base SI units are the ampere and the meter) units of S/cm historically have been selected as the accepted unit of expression.

At low ion concentrations (typically $<10^{-3}$ mole/L), the conductivity–concentration relationship is linear and valid because λ is constant for each ion, but there are three notable exceptions to this strict linearity and proportionality. First, at higher concentrations (approximately 10^{-3} to 1 mole/L) small negative deviations from linearity (<5% per decade) arise because of the decrease in λ for each ion, and the negative deviations vary from ion to ion. Second, at higher concentrations for weak acids and bases the extent of dissociation into ions decreases depending on their dissociation constants. As the concentration of a weak acid/base increases, the conjugate cation/anion concentration increases as the square root of the acid/base concentration. Third, at high concentrations (>20%) of certain strong acids such as HNO_3 and H_2SO_4, the negative deviations persist, and, in some cases, the conductivity decreases with increasing concentration. The conductivity of high-concentration acid systems is well documented.

Another variable that influences conductivity measurements is the fluid temperature. A stricter expression of *Equation 1* is shown below as *Equation 2*:

$$\kappa(T) = 1000 \sum_{i}^{all\ ions} C_i(T) \lambda_i(T)$$

where the conductivity measurement, ion concentrations, and specific ion conductances are temperature (*T*) dependent. As fluid temperature increases, the ions become more efficient electrical conduits, making this physicochemical phenomenon the predominant reason for the temperature-compensation requirement when testing conductive fluids. The specific ion conductance of all ions increases with increasing temperature. In addition, the concentration of ions can also change as a function of temperature. For example, the auto-dissociation constant of water, K_w, increases with temperature from 0° to 100°, resulting in the increased production of H^+ and OH^-.

Strictly speaking, it is challenging to temperature-compensate perfectly the conductivity measurement to a reference temperature unless the ionic species are well known. In many applications the ionic species are well known, and in most other cases simple assumptions make this issue less demanding. Temperature compensation is discussed in further detail in *Temperature Compensation*, below.

THEORY OF OPERATION

Alternating Current Measurement Method

Conductivity is measured by applying a voltage (or current) between two conducting electrodes and measuring the resistance of the fluid using Ohm's Law. Various methods are used to apply the voltage/current, but all have the property of using an alternating voltage/current (AC) in order to minimize polarization (or collection of ions) at the electrodes or any electrolytic reaction. If a direct voltage/current (DC) is used, then the positive ions will collect at the negative electrode, and negative ions will collect at the positive electrode. The collection of ions at the electrode prevents the flow of current and adversely affects the accuracy and stability of the conductivity measurement. The measuring frequency of the AC signal depends on the technology and can range from as low as 30 Hz in low-conducting fluids and up to 4 kHz in highly conductive fluids. The specific frequencies are not relevant to operation of the system because the drive frequency is embedded in the instrument's measurement systems and is integrally linked to the supplier's measurement technology. This chapter does not seek to evaluate different measurement technologies because they are usually microprocessor-controlled systems and are proprietary.

The two-electrode AC measurement technique is valid for use with all concentrations of ionic species ranging from acids and bases (high conductivity) to *Water for Injection* and *Purified Water* (low conductivity) and even to organic, weakly ionic species such as alcohols and glycols. The measurement can be sensitive to ion concentrations as low as 0.05 µg/L. For high ion concentrations, analysts can use an alternative 4-electrode measurement method in which the current is applied between two of the electrodes and the voltage is measured between the two other electrodes. Ions are driven to the current electrodes while the voltage electrodes make the measurement with limited polarization effects.

Units of Expression

There is no difference in the physical measurement of conductivity and resistivity—they are multiplicative inverse measurements of each other. Therefore, if one measurement is known, then the other value is readily calculated by taking the reciprocal of the numerical value and the units. There is also no difference in the instrumentation or the sensors. The only difference is how the measured value is reported or displayed for the convenience of the analyst. For example, 18.2 MΩ · cm

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= 0.0550 µS/cm and 5.23 kΩ · cm = 0.191 mS/cm = 191 µS/cm. Although the proper SI units are Ω · m or S/m, the traditionally used units are Ω · cm or S/cm.

Figure 1 shows relationships among conductivity, resistivity, and some example process fluids of various grades of purity. The conductivity of fluids in pharmaceutical systems varies over approximately 8 orders of magnitude. In high-purity water systems, the quantity of ions present in *Purified Water* or *Water for Injection* is very low, resulting in a conductivity <5 µS/cm and often approaching 0.055 µS/cm or less. [NOTE—The conductivity of the purest waters at temperatures less than 25° is less than 0.055 µS/cm.] In drinking waters, the conductivity may vary from 30 to 2000 µS/cm. In chromatographic separations, the conductivity of the eluent may vary from 0.1 to 100 mS/cm. For hot concentrated acids, the conductivity may be as high as 1 S/cm.

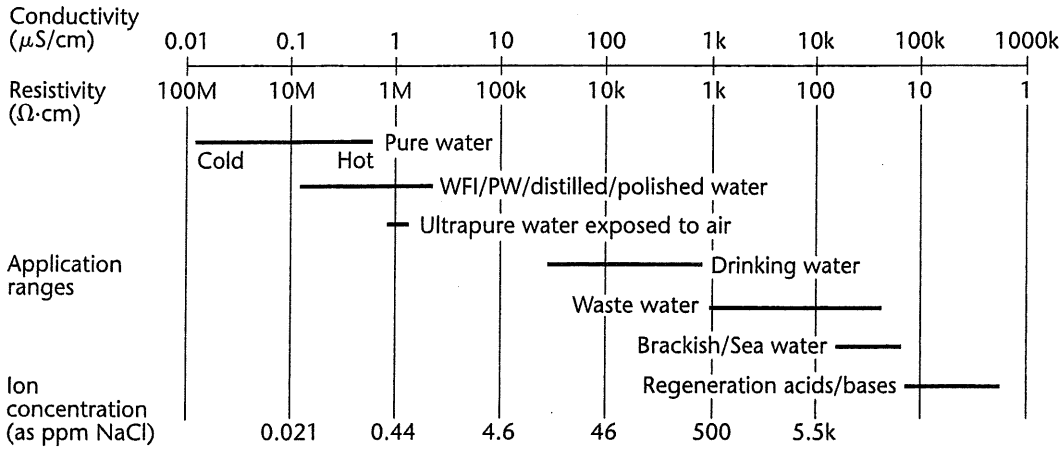

Figure 1. Relationships among conductivity, resistivity, and some example process fluids of various grades of purity.

Cell Constant Determination

The purpose of the sensor's cell constant is to normalize the conductance/resistance measurement for the geometrical construction of two electrodes. When two electrodes are placed in a conducting fluid and a voltage is applied to them, there is a conductance (resistance) between the electrodes. If the electrodes are placed farther apart, the conductance decreases (resistance increases). If the area of the electrodes increases, then the conductance increases (resistance decreases). In both cases, the ion concentrations between the electrodes do not change, but the geometrical construction of the sensor (cell constant) alters the measured conductance (resistance). The conductivity of a solution (κ, S/cm) is related to the conductance, G (siemens) according to *Equation 3:*

$$\kappa = G \times \left(\frac{d}{A}\right) = G \times \theta$$

where A is the area of the conducting electrodes (cm²) and d is the distance between the electrodes (cm). The other common unit of expression is the reciprocal of conductivity, or resistivity, ρ (Ω · cm), as described in *Equation 4*:

$$\rho = \frac{1}{\kappa} = \frac{1}{G \times \theta} = \frac{R}{\theta}$$

where R is the resistance of the fluid between the electrodes (1 Ω = 1 S^{-1}). The geometrical ratio, d/A (or θ), is known as the cell constant (cm^{-1}) of the sensor.

Determination of the cell constant by the direct measurement of d and A is impractical because of variations in the geometrical configurations and the nonuniformity of the electric field between the electrodes. Practically, the cell constant is determined by the measurement of aqueous solutions of known conductivity. See *Calibration* below.

Temperature Compensation

Temperature compensation is a typical requirement for most conductivity measurements, although there are exceptions such as those contained in *Water Conductivity* ⟨645⟩. As noted previously, the conductivity of a fluid is related to its temperature. As the temperature increases, ions become more mobile and the conductivity increases. The effect of temperature depends on the type and concentration of the ion, but for most solutions >10 µS/cm the impact of temperature is in the range of 1.9%/° to 2.2%/°. For strong acids, this may be as low as 1.5%/°. For high-purity compendial waters, the temperature coefficient varies from 2.0%/° to 7.5%/° depending on the temperature and purity of the water. For each case described here, some knowledge of the type of impurity is needed in order to ensure adequate temperature compensation. If the conductivity vs. temperature function is linear and the temperature coefficient is constant, the equation that relates the compensated conductivity to the non–temperature-compensated conductivity is described in *Equation 5*:

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$$\kappa_{25} = \frac{\kappa_T}{[1+\alpha(T-25)]}$$

where T is the measured temperature, κ_{25} is the conductivity compensated to 25°, κ_T is the conductivity at T, and α is 0.02 for a temperature coefficient of 2%/°.

Most conductivity measurement systems measure the uncompensated conductivity/resistivity and the temperature, and the temperature-compensated conductivity is determined via mathematical algorithms (e.g., by application of *Equation 5*) in the microprocessor of the transmitter. Depending on the application and knowledge of the content of the fluid, different compensation algorithms may be available. For most process control applications, temperature compensation is recommended because when the uncompensated conductivity changes it is impractical to distinguish whether this change is caused by temperature fluctuation or a change in ionic content. Temperature compensation allows the analyst to distinguish between changes in temperature and ionic content. Compensation to a reference temperature of 25° is standard practice, but some methods specify temperature compensation to 20°.

Most conductivity sensors have temperature devices such as a platinum RTD (resistance temperature device) or NTC (negative temperature coefficient) thermistor embedded inside the sensor, although external temperature measurement is possible.

OPERATIONAL CONSIDERATIONS

System Components

The usual components of a conductivity measurement system are the sensor, the transmitter, and the cable that connects the sensor to the transmitter. The sensor is the device that is in direct contact with the fluid. The sensor consists of the electrodes, usually an embedded temperature device, and a process connection (e.g., a tri-clamp if the sensor is intended for in-process sanitary applications). Traditionally, sensors are passive electromechanical devices and do not contain measurement circuitry.The transmitter is the device that measures the resistance between the sensor's electrodes, measures the resistance of the temperature-measuring device, converts the resistance measurement to a conductivity (or resistivity), and performs temperature corrections to compensate the signal to a reference temperature. The cable connects the sensor to the transmitter. In process control or monitoring systems, the cable allows the sensor to be attached to a process tank or pipe, and the transmitter can be located at a control panel or other remote location, if necessary. The distance between the sensor and the transmitter may affect measurement accuracy caused by the added cable resistance and the susceptibility of the cable to external noise. Therefore, this distance should be considered during selection of the measurement system. In some conductivity measurement systems, the measurement circuitry is directly attached to the conductivity sensor, allowing digital transfer of the measurement results to a remote display at greater distances than traditional measuring systems with wire transmission of analog signals.

Materials of Construction

The sensor's materials of construction are critical when the sensor may be in contact directly or indirectly with the product. For most laboratory applications, materials of construction are less critical. All sensors contain measuring electrodes and an insulating material between the electrodes. Technically, the only requirements are for electrodes to be electrically conducting and to be able to withstand the physical and chemical environment. Electrodes can be made of various grades of stainless steel, titanium, graphite, and many other metals. The insulating materials must isolate the electrodes from each other so that only the fluid conductivity is measured. Insulating materials are usually made of an inert polymer, epoxy, or ceramic.

If the sensor is connected directly to a process vessel or the piping system, then the sensor's materials of construction must meet the thermal and hydraulic (pressure) requirements of the process system. The sensor must have a proper process connection to the vessel/piping. Depending on the application, the sensor also may be required to meet biological compatibility, material, or hygienic design requirements. The sensor must not degrade during installation and operation. The sensor must be able to withstand any other processes that the system may encounter such as clean-in-place or steam-in-place processes. Otherwise analysts may need to remove the sensor from the process. When immersion of the sensor into the process places the process at risk, the sensor should be installed in a housing that is attached to the sidestream. In this case, a fraction of process fluid flows to the housing and sensor and then to drain. This allows indirect measurement of the process fluid without harm to the process or product.

If the conductivity measurement is performed off-line, e.g., in a laboratory environment, then the operating considerations are reduced because the thermal, hydraulic, and other product-contact concerns are reduced or eliminated.

CALIBRATION

The process of calibrating a complete conductivity measurement system generally consists of three parts. First, the transmitter's electronic circuitry is calibrated. Second, the temperature sensor device is calibrated. Third, the cell constant of the conductivity sensor is determined. In each case, verification may precede any calibration to determine if an adjustment is necessary. In most cases for microprocessor-controlled instrumentation, there is no formal mechanical or electronic adjustment of these subsystems. Instead, these adjustments are usually made in software-controlled calibrations that are computed automatically by the transmitter.

A calibrated transmitter should be used in order to calibrate the temperature sensor and cell constant. The transmitter used to calibrate the temperature sensor and cell constant can be the transmitter used in normal operation, or another transmitter can be used instead. Because of differences in a supplier's wiring and electronic compatibility, it may be necessary to use the same type of transmitter.

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As is the case for all instrument-based methods, calibration frequency depends on many factors. Depending on the type of conductivity sensor and transmitter, calibration cycles vary from weekly to annually based on manufacturers' recommendations, historical performance of the instrumentation, internal requirements, and the criticality of the application. For robust process instrumentation, typical calibration cycles for the electronics and the sensor take place approximately every 12 months. When the sensor's cell constant can be altered by the fluid or the process conditions, more frequent calibration may be needed.

Instrument Calibration

The transmitter's electronic circuitry is calibrated by disconnecting the sensor from the transmitter, connecting precision resistors (resistors of known value) to the transmitter, and comparing the traceable resistance value to the measured resistance value. The resistance values should be traceable to a competent national authority. The resistance values should be selected so that they are in the range of (1) the measurement capability of the transmitter, and (2) the resistance that will be measured during operation. The transmitter may have multiple circuits internally, so verification of the appropriate circuit (or all circuits) and measurement range is necessary. Comparison of the measured resistance to the actual resistance verifies if the transmitter is properly calibrated. The resulting difference must be within a predetermined x_1% of the actual resistance, where x_1 indicates the desired conductivity circuit electronics performance. Typical conductivity circuit electronics performance is usually in the range of 2% or less of the target value. Otherwise, adjustment of the resistance measurement circuit is recommended.

An example of the transmitter's measurement electronics calibration process follows: A typical operating range for a clean-in-place process may be in the 50 to 75 mS/cm range. A sensor with a cell constant of 5.0 cm^{-1} is used in this example. Based on *Equation 4*, this requires a measuring resistance of 67 to 100 Ω. To verify the conductivity measuring circuit during calibration, analysts should use a resistor(s) with a traceable value in or near this range. When compendial waters are used, typical measurements are in the range of 1 to 20 MΩ · cm and use sensors with a cell constant of 0.1 cm^{-1}. This computes to a resistance of 0.1 to 2 MΩ. Resistors with a traceable value in this range should be used. If the recorded values are within the range of the pre-established acceptance criteria (x_1%), then adjustment is not required.

Temperature Measurement and Sensor Calibration

If a temperature measurement circuit is integrated into the transmitter and is used as part of the measurement system, then verification and/or calibration of this circuit is required. Depending on the type of temperature device in the sensor, an appropriate signal source (e.g., resistance) should be input to the transmitter. Comparison of the measured temperature and the simulated temperature verifies if the temperature measurement circuit is properly calibrated. The resulting difference must be within x_2° of the simulated temperature where x_2 indicates the desired temperature circuit electronics performance. Typical temperature circuit electronics performance usually is in the range of ±1° or less. If the difference meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the temperature measurement circuit is recommended using the protocol in the transmitter's calibration function.

If a temperature sensor is integrated into the conductivity sensor, the temperature sensor can be calibrated by comparison of the sensor's temperature measurement to a reference system. This is accomplished by immersing the process and reference sensors in the same fluid. The reference system can be another traceable temperature measurement device or a fluid system of known temperature such as boiling water (corrected for elevation, if this degree of accuracy is necessary) or an ice-water bath.

The accuracy of the calibration is related to the accuracy of the reference sensor, the thermal homogeneity of the fluid, and the elimination or reduction of any artifacts that can negatively influence accuracy. For example, if the sensor is not fully immersed in the fluid, thermal conduction from the ambient environment can alter the temperature measurement. Careful insulation of the sensor from the ambient environment may improve the accuracy of temperature measurements, particularly if the reference temperature is substantially different than the ambient temperature.

Comparison of the measured temperature and the reference temperature verifies if the temperature sensor is properly calibrated. The resulting difference must be within x_3° of the reference temperature where x_3 indicates the desired temperature accuracy that is required for the process. Temperature sensor accuracy is usually in the range of ±2° or less. If the difference meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the temperature sensor calibration factors is recommended using the protocol in the sensor's calibration function.

Cell Constant Calibration

The cell constant of the sensor is determined by comparing the conductivity measurement of the system to that from a reference conductivity system and adjusting the cell constant. Place the sensor in a reference solution of known conductivity whose value is traceable to a competent authority. The reference solution can be a solution of known conductivity in one of three ways:

1. The solution can be produced according to a standard method that is traceable to a competent authority. One method is to prepare one of the solutions listed in ASTM D1125. Another method is to use ultra-pure deionized water with no exposure to air whose conductivity is known.
2. The solution can be procured from a third-party supplier with traceability to an acceptable authority. Cell constant calibration should be performed in the recommended temperature range of the reference solution.
3. The solution can be any fluid whose conductivity is known by measurement from an alternative and traceable reference conductivity measurement system.

In all three cases, regardless of the type of calibration methodology, there are some fundamental requirements. The sensor's cell constant should be calibrated at a conductivity that is in the measurement range of the measuring system. For example, if the system is designed to measure in the 0–100 µS/cm range, then use of a 1000 µS/cm reference solution is not advised.

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Because the cell constant is a geometrical property of the sensor and is a constant, the determination and/or calibration of the cell constant does not need to be in the operational range of the pharmaceutical process as long as the conductivity is in the operational range of the measurement system. Also, because the cell constant is a geometrical property of the sensor, a single-point calibration typically is sufficient. In some applications, a 2-point calibration can provide improved accuracy at very high conductivity. Depending on the reference solution, cell constant calibration can be performed with or without temperature compensation. For reference solutions exceeding 10 µS/cm, if temperature compensation is needed a temperature coefficient (α) of 2%/° is sufficient over the range of 25 ± 10° unless otherwise specified.

Adjustment of the cell constant is recommended if the difference between the measured and reference conductivity exceeds x_4% of the reference conductivity where x_4 indicates the conductivity accuracy that is required for the process. Typical conductivity accuracy is 5% or less. If the accuracy measurement meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the sensor's cell constant is recommended using the protocol within the sensor's calibration function.

OPERATION

In-line, At-line, and Off-line Measurements

Depending on the application, in-line, at-line, and off-line measurements have specific universal requirements. Each has advantages and disadvantages depending on the application, but the fundamental technologies (AC measurement) supporting each measurement type are constant. The primary differences between the in-line/at-line and the off-line systems are sensor features related to process robustness and transmitter features related to output functions, external communication capabilities, and system installation costs. In-line and at-line systems have added benefits of real-time measurements and continuous data acquisition. They also have added one-time electrical and plumbing installation costs, and the instrumentation is fixed in a specific location for a single process and purpose. Also, sample is diverted to drain, resulting in product loss. Note that the fluid flow velocity should be high enough that the velocity does not affect the measurement. Off-line (or laboratory-based) systems have the benefit of measuring many sample types under controlled conditions. Off-line batch testing has added costs associated with cleaning containers and collecting samples as well as risks of sample contamination.

In-line conductivity measurements are suitable when the following conditions are met: (1) there is a need for or value of real-time, continuous data; (2) the sensor and the application/fluid are compatible and cause no harm to each other; and (3) atmospheric contamination must be avoided. In these cases, continuous process control, decisions, and intervention are available.

At-line (or sidestream) conductivity measurements are used when the following conditions are met: (1) a need exists for or value of real-time, continuous data; (2) the sensor and the application are compatible with each other; (3) the sensor can cause harm to the application and/or fluid; and (4) atmospheric contamination must be avoided. In such cases the measurement is made by delivering the sample from the tank/piping using hydraulic pressure (flow) to the conductivity sensor, and the fluid passes through the sensor and goes to drain. In these cases, continuous process control, decisions, and intervention are available and have no affect on product or application. The only requirement is positive pressure upstream from the sensor to prevent backstreaming of the sample fluid back into the process.

Off-line conductivity measurements are used when the following conditions are met: (1) no need exists for or value of real-time or continuous data; (2) the sensor and the application are compatible with each other, but the sensor can cause harm to the application and/or fluid; and (3) sampling needs are infrequent or are needed on a limited basis.

Sample Preparation and Off-line Measurement

When performing off-line measurements, analysts must ensure that the container used to collect the fluid sample(s) is sufficiently clean so that the container does not alter the result. Collect an amount of fluid needed for rinsing and measuring. For samples such as *Purified Water* where the analysis can be affected by gasses, containers should be filled completely to reduce headspace. The volume of fluid needed to make a measurement depends on the design of the sensor and can range from <10 mL to 1 L. Transfer a portion of the sample fluid to the clean measuring container to rinse the container walls. Discard the fluid. The sample container also can be used as the measuring vessel. The sensor should be rinsed with a suitable quality of water or other appropriate fluid before use so the measurement is not affected and then rinsed at least once with the solution to be measured, which then is discarded. This rinse with sample solution removes any residue or fluids with which the conductivity sensor previously was in contact. The exact procedure varies depending on the solution tested. For example, when testing very low conductivity samples such as *Purified Water*, analysts must do more rinsing to remove any remaining residue from the sensor. Maximum holding time, temperature, and container type must be controlled for some low-conductivity samples, e.g., high-purity waters, because of the potential impact of ionic leachables from certain containers.

Transfer the fluid to the measuring container, immerse the sensor into the fluid, and ensure acceptable clearance around the sensor's measurement area. Stir or agitate the sample to prevent bubbles from attaching to the electrodes and disturbing the measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm in the transmitter, and adjust the sample temperature to the recommended range, if necessary, using an appropriate temperature bath. Otherwise, disable the temperature compensation. Verify that the temperature is sufficiently stable (<0.25° change per min), and record the conductivity reading and the temperature, if necessary.

In-line Measurement

For in-line testing, the sensor is installed into the process piping or vessel. The orientation of the sensor relative to the flow of the fluid is critical to ensure that (1) particles and sediment do not collect between the measuring electrodes, and (2) no air

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pockets are trapped between the measuring electrodes. Both factors can adversely affect the measurement accuracy. Depending on the electrode design, manufacturers usually provide recommendations. Ensure the sensor is clean before installation.

After the sensor is immersed in the closed process, such as a piping system or tank, the sensor does not require removal and cleaning before a measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm. Otherwise, disable the temperature compensation. Although temperature stability is desirable, it may not be possible depending on the temperature control of the process. Record the conductivity reading and the temperature, if necessary.

At-line Measurement

For at-line testing, a piece of tubing is used to connect the process vessel/piping to the sensor and its housing. The tubing should be cleaned or flushed with an appropriate cleaning agent to remove any impurities that could alter the conductivity measurement. The tubing can be made of metal or plastic depending on the application and chemical compatibility. Installation of the sensor into a housing permits fluid to be directed into the sensor and then to drain. Installation considerations are similar to those for in-line installations. Ensure the sensor and housing are clean before installation.

After the sensor and housing are connected to the sidestream, the sensor does not require removal and cleaning before a measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm. Otherwise, disable the temperature compensation. Although temperature stability is desirable, it may not be possible depending on the temperature control of the process. Record the conductivity reading and the temperature, if necessary.

Other Considerations

In all cases mentioned above, the use of abrasive materials to clean the sensor typically is discouraged. Two reasons to avoid abrasive cleaning materials are that (1) the passive layer of stainless steel sensors (when used) can be destroyed, and (2) the surface of the measurement area can affect the measurement accuracy. For these reasons, the use of appropriate chemically compatible fluids is preferred instead of mechanical methods for cleaning.

Unlike the requirements for many electrochemical measurements, the need for flowing, circulating, or agitating fluid is not a fundamental requirement for a conductivity measurement. In general, there is no difference in the conductivity measurement of a static or a flowing sample, but two conditions must be met for static samples: First, bubbles cannot be allowed to collect on the measuring area of the electrodes because they can interfere with the flow of current conductivity measurement. Second, if sample homogeneity is affected by the lack of agitation or circulation, then the conductivity measurement may not represent the conductivity of the bulk fluid.

In all cases, the installation of the sensor should take into consideration wall effects from the vessel, piping, or laboratory container. If the proximity of the wall interferes with the electromagnetic field for the conductivity measurement, then the measurement could be positively or negatively altered. Some 2-electrode sensor designs, such as coaxial concentric electrodes, are not affected by nearby objects. The sensor's installation instructions may indicate if this must be considered.

If unstable conductivity readings are observed, some common causes could be inadequate grounding of the water system, electronic noise from pumps and other high-frequency generators, or internal leakage of the sensor. Various diagnostic approaches are available and can help identify the cause.

⟨1660⟩ EVALUATION OF THE INNER SURFACE DURABILITY OF GLASS CONTAINERS

PURPOSE

This general information chapter provides information about factors that affect the durability of the inner surface of glass containers. Recommended approaches are provided to evaluate the potential of a drug product to cause formation of glass particles and delamination of the inner surface. Screening methods are provided to detect glass particles and delamination, allowing a comparison to be made of glass durability on a lot-to-lot basis or between different glass manufacturers.

SCOPE

This chapter addresses bottles and vials manufactured by molding and ampuls, cartridges, vials, and prefillable syringes manufactured from tubing glass. Glass for pharmaceutical packaging is classified as Type I borosilicate glass, Type II treated soda-lime-silica glass, or Type III soda-lime-silica glass on the basis of the hydrolytic resistance of the glass, as defined in *Containers—Glass* ⟨660⟩. Type I glass containers are suitable for most products for parenteral and nonparenteral use. Type II glass containers are suitable for most acidic and neutral aqueous products for parenteral and nonparenteral uses, and can be used for alkaline parenteral products when stability data demonstrate their suitability. Type III glass containers usually are not used for parenteral products or for powders for parenteral use, except when suitable stability test data indicate that Type III glass is satisfactory. This chapter focuses primarily on Type I glass, because it is the most widely used in the pharmaceutical and biopharmaceutical industry for parenteral products although the guidance can be equally applied to Type II and Type III glass used for parenteral products.

The chapter should be useful for the following:

- Molded and tubular glass container manufacturers and converters

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- Pharmaceutical and biopharmaceutical companies
- Contract manufacturing and filling organizations

Glass delamination may be described as the appearance of thin flexible flakes of glass (or lamellae) that can range in size from <50 µm to 200 µm in a drug product solution. This is a serious quality issue and can result in a product recall. The appearance of glass lamellae is a lagging indicator of a strong interaction between the drug product and the inner surface of the glass. Although delamination is the most obvious visual indicator, it represents the final stage of a complex glass corrosion reaction, and can be observed only at a point where prevention is no longer an option. Adding further complexity to detection, mechanical energy from shaking or vial-to-vial contact during transportation may be required to dislodge the lamellae from the internal surface of a filled vial and facilitate observation.

Tests for delamination combine the visual examination of the solution, an examination of the vial's internal surface and analysis of an aggressive test solution to assess the propensity of the internal glass surface of vials to delaminate. These examinations and the use of an aggressive test solution are intended to be conducted by the pharmaceutical manufacturer, not the glass manufacturer or converter.

GLASS TYPES

Glass in its pure form consists of silicon dioxide with a melting point in excess of 1700°. However, this is rarely used commercially because of the cost of working at these elevated temperatures. Added network modifiers, such as sodium, potassium, or boron oxide, lower the melting point and lower the chemical durability, whereas added network stabilizers, such as calcium and aluminum oxides, improve the durability of the glass. Colored glass (e.g., amber glass) is produced by transition metal oxides such as iron oxides. All additives to pure silicon dioxide, as well as silicon itself, can be viewed as potential extractables from glass containers.

Glass compositions do not exist as stoichiometric chemical compounds but rather are expressed by a range of compositions. Thus, there is allowable variation within a glass type, and glass types may vary slightly among glass producers. Soda-lime-silica glass consists of silicon dioxide (60–75 wt%), sodium and potassium oxides (12–18 wt%), and smaller amounts of calcium, magnesium, and aluminum oxides (5–12 wt%). This glass has a relatively high coefficient expansion (COE) of 80–90 × 10^{-7} per degree and is susceptible to breakage by thermic shock. Borosilicate glass consists of silica (65–80 wt%), boric oxide (7–13 wt %), and smaller amounts of sodium, potassium, and aluminum oxides. The presence of boron provides greater resistance to thermal shock through a reduction in COE and to hydrolytic attack by increasing the connectivity of the glass network. Type I glass is available in multiple formulations: tubular glass is available with a low COE, described as 32–33 expansion glass and with a relatively low COE (range, 48–56 expansion), for example 51 expansion glass, in reference to their individual COEs of 32.5 × 10^{-7} per degree and 51.0 × 10^{-7} per degree, respectively. Molded glass has a higher COE in the region of 60–63 expansion.

FORMATION OF MOLDED AND TUBULAR GLASS CONTAINERS

Formation of molded and tubular glass containers requires a number of steps. The quality of the container used in packaging depends on the conditions and the quality control of each step. Both molded and tubular containers originate from a glass furnace, and different furnaces are dedicated to borosilicate or soda-lime-silica glass. The refractory bricks lining the furnace deteriorate with time and must be replaced. Worn bricks can contribute to cosmetic defects such as stones (inclusions in the glass) that become incorporated into the molded glass containers or glass tubing.

Molded glass vials and bottles are manufactured in a one-step process whereby a stream of molten glass is cut into a gob, which then enters a mold where air or tooling is used to shape the container to the mold. Formation of containers from tubing glass is a two-step process. Glass tubes of a specific diameter are formed from a stream of molten glass that exits the furnace, is cooled, and is sectioned into standard lengths. These tubes are subsequently converted into glass containers (ampuls, cartridges, syringes, or vials) by either the tubing glass manufacturer or by independent converters. It is technically difficult to form glass tubing with a diameter sufficient to make bottles containing 100 mL or more, so these containers are produced by molding.

Gas flames are used to soften tubing glass to form the neck, to melt the glass to form the base of ampuls or vials, and to separate the container from the glass tube. In the case of cartridges and prefillable syringes, the glass tube is cut to length, and the ends are softened to form the nozzle and flange of the syringe and the neck and rear of the cartridge. Heating rate, maximum glass temperature, and production speed are critical parameters that can be adjusted for individual forming machines. After formation, both tubular and molded containers pass through an annealing oven (lehr) that heats the containers to 20° to 30° above the transformation temperature (T_g) of the individual glass formulation (T_g for borosilicate glass is approximately 570°) and then gradually cools them in order to remove stresses in the container due to the manufacturing process. This too is a critical process because poorly annealed containers show reduced chemical and mechanical durability.

The process of forming tubular vials and ampuls has an effect on the local surface composition of the glass. During formation of the neck and particularly the base, the temperature of the inner surface of the containers can exceed the evaporation point of some of the glass components such as alkali borates. Under certain time–temperature conditions, the glass can phase separate during forming, creating nonhomogenous surface chemistry on the interior of the container. Both scenarios are undesirable for the storage of aggressive liquids from a surface chemical durability perspective. Evidence of this can be obtained by appropriately etching the glass with acid, after which an opaque ring will appear above the heel of the container, indicating a negative change in the inner surface chemistry. The same phenomenon can be observed at the shoulder of the container as well, but in many instances this area does not experience prolonged contact with a liquid.

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PROCESSING OF MOLDED AND TUBULAR GLASS CONTAINERS

At times, the inner surfaces of glass ampuls, vials, and bottles undergo additional treatments. As an example, heating glass propagates sodium oxide toward the inner surface of the container, but washing with water does not remove sodium oxide because of the latter's limited solubility. When glass is exposed to an aqueous solution, sodium ions diffuse into the solution from the glass surface to produce hydroxide ions, resulting in an elevated pH in unbuffered solutions. One common treatment is the use of ammonium sulfate which converts the sodium oxide on the inner surface to a depth of approximately 10–100 nm into highly soluble sodium sulfate that then can be removed by washing. Although removal of sodium ions from the surface does reduce the propensity for pH shift, the treatment does remove structural elements, leaving a thin silica-rich inner surface layer. The process originally was designed to raise the surface hydrolytic resistance of Type III soda-lime-silica glass to that of Type II glass in order to mimic the hydrolytic resistance of Type I glass. This process also can be applied to Type I glass.

In summary, the key factors that influence glass surface durability of containers manufactured from Type I glass are primarily the manufacturing conditions, such as the forming temperature, the time of exposure to heat, and the annealing conditions. The temperatures used for subsequent steps are lower than those used for forming and annealing (see *Table 1*), and do not pose an additional risk to the chemical durability of the glass from phase separation or volatilization. Post-manufacturing operations such as storage in humid conditions and processing, such as depyrogenation in the presence of water vapor and terminal sterilization via autoclaving, can also impact glass surface chemical durability.

Table 1. Temperatures Encountered During Formation and Processing of Type I Tubular Glass Containers

Key Operations	Typical Temperatures (°)
Furnace	1500–1650
Sectioning of tube and base formation	1300–1500
Working range	1000–1250
Softening	750–850
Annealing	550–600
Depyrogenation range	250–350
Terminal sterilization	110–130

GLASS CONTAINER SOURCING

A pharmaceutical manufacturer has a range of choices when selecting a glass container for a drug product. These include the type of glass (I, II, or III), the production method (tubular or molded), surface treatments, as well as the size and neck finish of the container. It is important that the pharmaceutical manufacturers provide sufficient information on their requirements, such as the drug product formulation and the manufacturing and filling process, to allow the glass vendors to make informed judgments as to what containers to recommend.

Pharmaceutical manufacturers should consider the upstream provenance of the containers they purchase in that they should have sufficient knowledge of the glass manufacturing process and glass composition. This is essential to qualify a particular glass container type from a glass manufacturer for a particular drug product. The following knowledge is useful in this regard:

- Glass formulation
- COE for Type I tubular glass (32–33 or 48–56 expansion)
- Whether the glass converter makes its own glass feedstock or sources the glass feedstock from a third party
- The manufacturing site for the glass containers. If multiple sites manufacture glass containers for a given product, information to determine if the glass containers made at the different sites will perform comparably
- Whether the glass surface has been modified through chemical treatment such as ammonium sulfate by the glass manufacturer or converter.

The maker and user of a glass container should collaborate to assure that glass quality is monitored and maintained throughout the extent of the glass supply relationship. Glass quality should also be monitored and inferred by the user through observations made during storage throughout the product's use-by-date. The glass manufacturer and glass user quality management programs should include the following:

- Quality audits of glass supplier (glass manufacturer and/or converter) by the glass user
- Establishing mutually agreed upon acceptable quality levels for lots of glass containers
- Monitoring and trending of the quality of glass batches, including but not necessarily limited to, monitoring the values obtained by the *Surface Glass Test* in ⟨660⟩
- Monitoring and trending of glass quality and glass manufacturing process performance by the glass container manufacturer, including the effectiveness of methods used during the manufacturing process of the glass container to measure geometric tolerances and identify cosmetic defects
- Assurance that differences among different glass manufacturing sites do not significantly affect quality of a specified glass container sourced from multiple sites
- Presence of a system to monitor and qualify changes made to the glass manufacturing process and to inform customers of such changes.

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GLASS SURFACE CHEMISTRY

After manufacturers are assured of the quality and consistency of the glass containers they purchase, they can use the complex aqueous chemistry of surface glass to decide on potential drug product formulation and treatment steps that could increase glass stability. The first reaction between the glass surface and an aqueous phase (water or water vapor) involves ion exchange between hydrogen ions (or hydronium ions H_3O^+) from the aqueous phase and alkaline ions in the glass (*Equation 1*). This ion-exchange occurs in a short reaction time in acidic or neutral solutions. In basic solutions, the reaction occurs at the glass/water interface and dissolves the silica network. Further reaction of the silica releases silicic acid into the solution, thereby lowering the pH (*Equation 2*). These reactions result in hydration of the glass surface and an alkali-depleted, silica-rich layer.

$$H^+ + Na^+SiO^- \text{ (glass)} \Rightarrow SiOH + Na^+ \qquad [1]$$

$$H_2O + Na^+SiO^- \text{ (glass)} \Rightarrow SiOH + Na^+ + OH^- \qquad [2]$$

The presence of water in the leachate promotes hydrolysis of the Si–O bond forming a silica-gel layer (*Equation 3*).

$$H_2O + Si\text{-}O\text{-}Si \Leftrightarrow 2\ SiOH \qquad [3]$$

The mechanical properties of the surface gel that forms are different from those of bulk glass. Repeated hydration and dehydration of the layer leads to the cracking of the gel layer and eventual generation of particles. This process is worsened as the gel layer increases in thickness. This phenomenon is well known in glass exposed to ambient moisture (known as weathering). At higher pH values, the mechanism of glass degradation changes from the leaching of alkali elements to the dissolution of the silicate network as shown in *Equations 4* and *5*.

$$SiO_2 + 2H_2O \Leftrightarrow H_4SiO_4 \qquad [4]$$

$$H_4SiO_4 + OH^- \Leftrightarrow H_3SiO_4^- + H_2O \qquad [5]$$

Reaction (*Equation 5*) increases the solubility of the silicic acid in solution, driving the reaction forward. At some point the limit of solubility is exceeded, and particles are formed via precipitation. If the solution is not buffered, a decrease in the solution pH will take place. These reactions and scenarios apply only to the reactions of glass with water; the presence of drug product formulations can complicate the situation considerably.

FACTORS THAT INFLUENCE INNER SURFACE DURABILITY

A number of factors have the potential to negatively influence the chemical durability of the inner surface of glass containers. These factors include glass composition, the conditions under which the containers were formed, subsequent handling and treatments, and the drug product in the container (*Table 2*). Not only can an aggressive drug substance corrode the inner surface, but excipients such as buffers, chelating agents and organic acids and high pH can also have a deleterious effect. For example, neutral solutions of sodium citrate attack glass with a severity similar to that of substantially alkaline solutions. Organic acids, such as gluconic and malonic acids, also corrode glass through a proposed mechanism of an ion exchange reaction in which metal ions on the glass surface are replaced by hydrogen ions from the acid. Not all listed factors negatively influence surface durability to the same degree, and can contribute to delamination either acting alone or in combination. Because of the range of variables, end users should examine all relevant variables for an individual drug product and assess the degree of risk for delamination and formation of subvisible and visible glass particles. In some situations, the accumulation of risk factors may indicate that the selection of a glass container for a particular formulation should be done following a predictive screening study to establish more stringent glass quality requirements or may indicate that a glass container should not be used for a formulation.

Table 2. Factors That Influence the Inner Surface Durability of Glass

Container Manufacture	Container Processing and Storage	Drug Product: Formulation, Processing, and Storage
• Glass composition • Molded or tubular container • Tubular manufacturing process: — Converting speed — Converting temperature	• Post-formation treatments: — Ammonium sulfate —Washing —Depyrogenation • Storage conditions: — High humidity	• Drug substance • Formulation: — Acetate, citrate, phosphate buffers — Sodium salts of organic acids, e.g., gluconate, malate, succinate, tartrate — High ionic strength, e.g., >0.1 M of alkaline salts — Complexing agents, e.g., EDTA — High pH, e.g., >8.0 • Terminal sterilization • Labeled storage conditions (refrigerated or controlled room temperature) • Shelf life

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EVALUATION OF THE INNER SURFACE DURABILITY

Each lot of Type I, II, or III glass containers received by a pharmaceutical manufacturer must comply with the *Surface Glass Test* in chapter ⟨660⟩. This test provides an indication of inner surface chemical durability but does not appear to provide a clear direct correlation with the propensity to form glass particles or to delaminate. The alkalinity value represents the sum of all the internal surfaces of the container, and although this is representative for molded containers, tubular glass vials can have different degrees of surface chemical durability, depending on the location (e.g., just above the heel versus the side wall). A low surface alkalinity value can be obtained from containers treated with ammonium sulfate but the treatment itself may reduce the inner surface chemical durability, dependent upon the drug product formulation used to fill the vial. The most important variable that affects the surface durability is the drug product itself, and because it uses water as the extracting medium, the *Surface Glass Test* does not take this into consideration. Therefore, the *Surface Glass Test* represents only a first step in quality control of surface chemical durability, and additional screening methods should be used to demonstrate the suitability of vials for a formulation from a particular source before formal stability studies begin.

Predictive Screening Methods

Screening methods help evaluate glass containers from different vendors (molded or tubular), glass formulations (e.g., 32–33 or 48–56 expansion for tubular glass), and post-formation treatments. Screening also establishes lot-to-lot variation from individual vendors during the drug development process, as well as lot-to-lot variations for products that have been shown to have a particular propensity to form glass particles or to delaminate. Screening methods can use a number of different technologies to examine three key parameters: visual examination and chemical profile of the inner surface layer, the amount and identity of extracted elements in solution, and the number of subvisible and visible particles in solution. Taken together, these elements are assessed by predictive tests for formation of glass particles and delamination, processes that reflect reduced durability. Predictive tests should look for precursors that lead to delamination rather than looking only for glass lamellae, and should be able to quickly provide predictive indication of surface durability. This makes the tests useful not just for vendor selection but also for evaluation of individual lots if necessary. Some of the more commonly used analytical techniques for evaluating the three key parameters are shown in *Table 3*.

Table 3. Analytical Techniques for Screening Studies

Parameter	Test Parameter	Instrumentation
Glass inner surface	• Degree of surface pitting • Chemical composition as a function of depth	• DIC Microscopy[a] or EM[b] • SIMS[c]
Extracted elements in solution	• Conductivity/pH • SiO_2 concentration	• Conductivity/pH meter • IC–MS[d] or ICP–OES[e]
Lamellae and visible and subvisible glass particles	• Presence of lamellae and visible particles • Lamellae or particle number and size • Lamellae or particle morphology and composition	• Visual inspection • Particle size analyzer • SEM–EDX[f]

[a] Differential interference contrast microscopy.
[b] Electron microscopy.
[c] Secondary ion mass spectrometry.
[d] Inductively coupled plasma–mass spectrometry.
[e] Inductively coupled plasma–optical emission spectrometry.
[f] Scanning electron microscopy–energy-dispersive X-ray spectroscopy.

Aggressive Screening Conditions

In selecting an appropriate primary glass container for pharmaceutical liquids, analysts should consider two approaches. The first is a series of accelerated temperature exposures using aggressive conditions that establish, in rank order, the chemical durability of the container without any specific reference to a given compound. Such testing can be helpful when selecting a packaging system for which the most chemically durable glass is desired. This testing also can be helpful in determining if changes in glass quality have occurred or in assessing processing changes that have been made by the primary container manufacturer. *Table 4* provides three examples of model systems that could be used for this assessment. Other model systems may be developed by the end users.

Table 4. Formulations and Conditions Used to Accelerate Delamination

Formulation	0.9% KCl pH 8.0	3% Sodium Citrate pH 8.0	20 mM Glycine pH 10.0
Conditions	1 h at 121° 1 or 2 cycles	24 h at 80°	24 h at 50°

Screening Strategy for Drug Products

Indicators include the appearance of a pitted, fractured inner surface particularly around the heel of the vial instead of a smooth surface, as well as a number of changes in the test solution, especially increases in SiO_2 concentration, the number of subvisible particulates in the solution, and a change in pH.

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If the purpose of the glass screening is to determine the suitability of a given glass container for a specific product, the testing proposed in *Table 4* is insufficient. The exposure conditions are too harsh and do not provide a direct link to the product itself. In these instances, accelerated conditions are still relevant, but they must link to the relevant conditions for the given product. For example, if a product will be stored at 5° and the appropriate accelerated conditions are 30°, then testing should occur at 30°. Many products or formulations cannot withstand the elevated temperatures or high pH shown in *Table 4*. In addition, false positive testing results could be obtained because the unusually high temperatures shown in *Table 4* could cause signs of delamination, but moderate exposure at 30° would produce no evidence of glass incompatibility.

Because lower temperatures are required for actual product testing, the duration of testing must be longer, ranging from weeks to months. A larger number of vials also is appropriate for this scenario because the goal of the testing is to ensure the results are representative of the quality of the glass that will be used for the drug product. *Table 5* shows some of the conditions that could be used for testing with a specific product.

Table 5. Screening Strategy for Glass Vials

Stress Test	Water Control	Drug Product Control
• Vials: washed, depyrogenated • Filled with Stress Test solution • Accelerated time and temperature treatment conditions	• Vials: washed, depyrogenated • Filled with Water for Injection • Autoclave if applicable to Drug Product • Accelerated Drug Product stability storage conditions	• Vials: washed, depyrogenated • Filled with Drug Product • Autoclave if applicable • Accelerated Drug Product stability storage conditions

CONCLUSIONS

Evaluation of the internal surface of glass containers begins with the *Surface Glass Test*, which uses water as the extracting medium. A low value is not always an indicator of a durable inner surface if the results are obtained using surface treatments (e.g., ammonium sulfate). Such treatments can lead to a silica-rich inner surface layer that represents a weakened glass structure, and risk of delamination increases when the vial is filled with formulations that contain aggressive agents such as organic acids, EDTA, or solutions that have high ionic strength or high pH. The screening methods and strategies described in this chapter can assist in the evaluation of glass containers from different suppliers or on a lot-to-lot basis and can provide an indication of the propensity of the selected formulation to cause delamination over time. Selection of glass vials intended to contain a drug product with one or more of the formulation risk factors identified in *Table 2* should undergo particular scrutiny.

⟨1661⟩ EVALUATION OF PLASTIC PACKAGING SYSTEMS AND THEIR MATERIALS OF CONSTRUCTION WITH RESPECT TO THEIR USER SAFETY IMPACT

INTRODUCTION

Drug products can chemically interact with their associated packaging systems and/or the system's plastic materials and components of construction while the product is being manufactured, shipped, stored, and administered. The magnitude of these interactions must not be such that the interactions adversely affect the suitability for use of the drug product or the packaging system. While suitability for use includes several quality aspects of the packaged drug product and its performance, the suitability for use aspect addressed in this chapter is patient safety.

The potential patient safety impact of interactions between a drug product and its packaging is assessed and established via the appropriate testing of the packaging systems and its materials and components of construction. *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩ establishes the tests and specifications that are necessary and appropriate for ensuring that such systems are suitable for use, specifically safe for use. Chapter ⟨661⟩ consists of two sub-chapters, *Plastic Materials of Construction* ⟨661.1⟩ and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩.

SCOPE

The purpose of this chapter is to communicate the key concepts behind ⟨661⟩ and its related sub-chapters, ⟨661.1⟩ and ⟨661.2⟩, and to provide additional information and guidance regarding the application and applicability of this set of chapters. Given the large and diverse nature of the pharmaceutical marketplace, the proper use and application of the ⟨661⟩ suite of chapters may not be intuitive to some stakeholders. Therefore, this chapter is intended to assist users in understanding and utilizing these chapters.

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GENERAL PRINCIPLES

The Overall Assessment Process

The objective of *USP* packaging systems standards is to establish the tests and specifications that ensure packaging systems do not materially impact the safety or effectiveness of pharmaceutical products. Given the complex nature of packaging systems and their manufacturing and development processes, multiple testing procedures are needed to establish their suitability for use with a specific pharmaceutical product. The logical development and manufacturing process progression for packaged drug products, starting with the packaging system's materials of construction, continuing with the packaging system itself, and ending with the packaged drug product, forms the basis of a three-stage approach to packaging systems qualification, as illustrated in *Figure 1*.

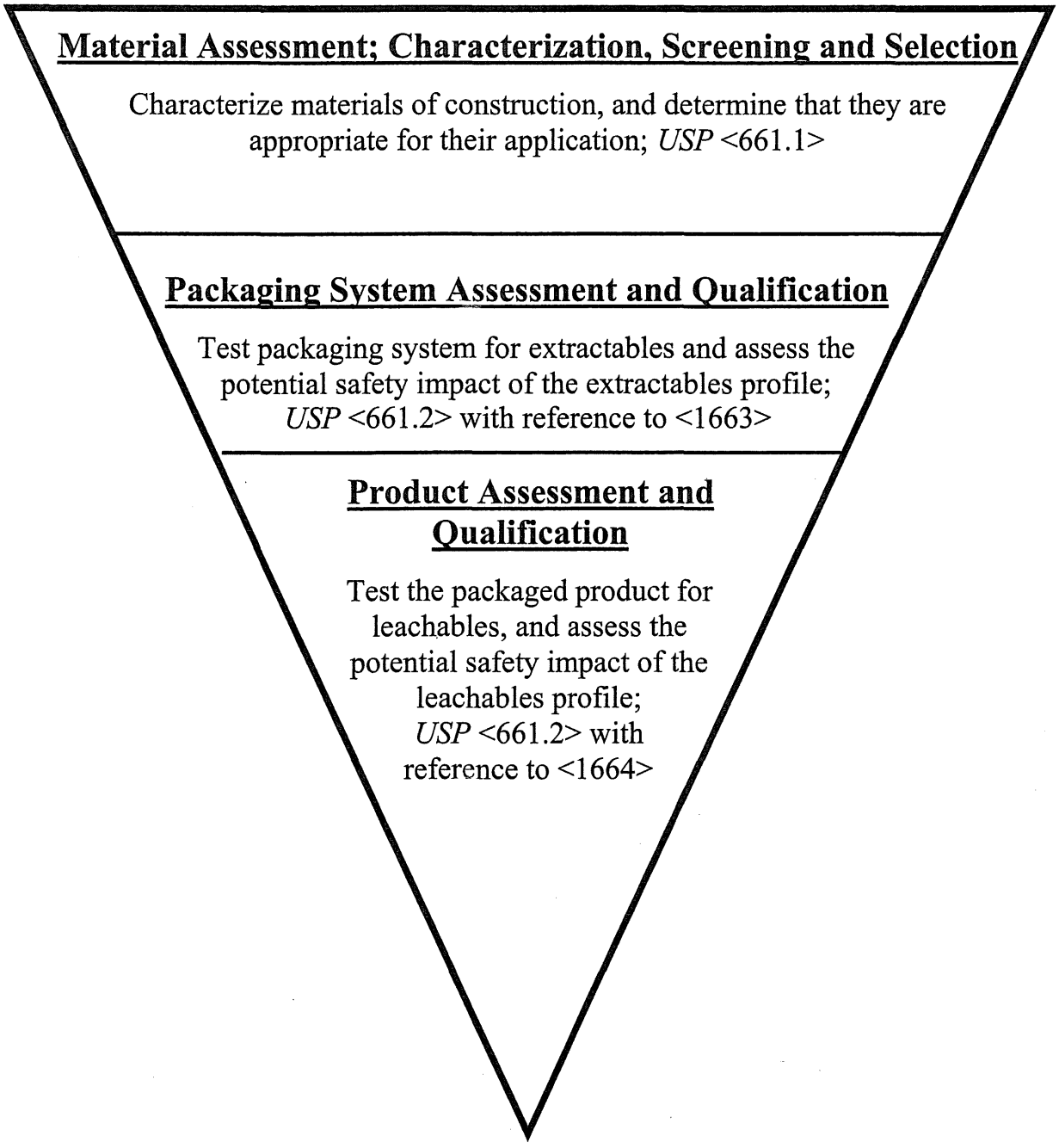

Figure 1. The three-stage process for the characterization and safety qualification of packaging systems and their materials of construction.

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The process for establishing a packaging system's suitability for use includes: characterization of its materials of construction (ingredients); testing and assessment of the system itself (extractables); and testing and assessment of the packaged pharmaceutical product (leachables). The initial step of the process involves chemically characterizing candidate materials of construction to the extent that the choice of materials to use in the construction of a packaging system can be rationally made and scientifically justified. The intermediate step of system assessment is useful and necessary as it bridges the risk assessment gap between testing starting materials and testing finished product, while providing a means for optimizing pharmaceutical product testing. The intermediate test is necessary as materials of construction undergo considerable stress, such as exposure to high temperatures, while they are being converted into either components of the packaging system or the packaging system itself. Furthermore, processing aids and additional additives may be introduced during the manufacturing process for a packaging system. Thus, the extractables profile of a system is likely to be different from, and potentially more complex than, the sum of the extractables profiles of its materials of construction. Therefore, the initial assessment of risk made in material selection is appropriately revisited by testing and qualification of the overall packaging system itself.

Ultimately, the effect that packaging may have on the drug product user is mediated by packaging-derived substances that are present in the drug product. The third stage of the process is product assessment, specifically leachables testing of the packaged product and impact assessment, which considers the user's exposure to the leachables.

Materials Assessment: Characterization, Screening, and Selection, *USP* ⟨661.1⟩

To ensure that a packaging system is suited for its intended use, it is important to select materials of construction which are suited for use in packaging systems. Testing and characterizing materials of construction for attributes relevant to their suitability provides a rational basis for material selection in designing a packaging system. The intentional selection of well-characterized materials minimizes the risk that a system made from those materials will be unsuitable. Considering safety specifically, selection of materials that have the tendency to be safe increases the likelihood that packaging systems made from those materials will be safe. Therefore, the characterization of materials of construction is the first step in the process of developing and qualifying safe packaging materials. Additionally, chemical characterization data may also provide the basis for effective and appropriate change control.

The intent of ⟨661.1⟩ is to establish, with a degree of confidence, whether potential material candidates could adversely affect the quality and safety of pharmaceutical products. The basic tenet of materials assessment, as reflected in ⟨661.1⟩, is that knowing the general composition and certain general characteristics of a material of construction allows one to:

- Rationally assess the potential safety impact of the materials with a degree of certainty that is appropriate for early product development and/or manufacturing.
- Forecast with some degree of accuracy the identity of the extractables from that material of construction and from systems that use that material of construction.
- Use the assessment and forecast to establish and justify the use (or non-use) of a particular material in a particular packaging system.

To this end, ⟨661.1⟩ defines a well-characterized material of construction as one whose:

- Identity has been definitively established.
- Biocompatibility (biological reactivity) has been established.
- General physicochemical properties have been established.
- Additives and extractable metals have been quantified.

Chapter ⟨661.1⟩ testing is not a guarantee that plastic systems constructed from materials meeting ⟨661.1⟩ specifications will be suitable for their intended use as it is not always the case that testing of a system's materials of construction directly and completely correlates with subsequent testing of the plastic system. Characterization of a material using ⟨661.1⟩ merely establishes the composition or characteristics of the material and enables the decision as to whether the material is an appropriate candidate for use in a packaging system. Nevertheless, ⟨661.1⟩ testing leverages the logical connection between material additives, material extractables, and system extractables, and thus provides a useful indication of the probable suitability-for-use issues for materials and systems. The actual qualification of the material occurs when the entire system is qualified for use in a particular application via ⟨661.2⟩ testing.

Packaging System Assessment and Qualification, *USP* ⟨661.2⟩

The impact of packaging systems on the chemical composition of packaged drug products can be established in two ways. The packaging system itself can be characterized with respect to substances that can be extracted from it (extractables). Secondly, the packaged drug product can be tested for packaging-derived substances that have leached into it (leachables). In the case of extractables assessment, the impact is predicted based on a relationship which is established (or inferred) between extractables and leachables. In the case of leachables assessment, the impact is specifically measurable, assuming that all the relevant leachables can be discovered, identified, and quantified in the packaged product. In either case, ⟨661.2⟩ establishes the tests and specifications for the packaging system, while referring users to relevant informational chapters (e.g., *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ for extractables and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩ for leachables) for insights on how to design and execute relevant studies.

Considering the packaging system as the test article, the intent of ⟨661.2⟩ is to define and delineate the testing needed to produce the data required for establishing the packaging system's safety. Chapter ⟨661.2⟩ refers to this process of establishing the safety of packaging systems as chemical assessment and notes that a packaging system is chemically suited for its intended use if:

- The packaging system is constructed from well-characterized materials, as established by testing according to ⟨661.1⟩.
- The packaging system's general physicochemical properties have been established.

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- The packaging system's biocompatibility (biological reactivity) has been established.
- The packaging system has been established to be safe by means of the appropriate chemical testing and toxicological assessment.
- The packaging system is chemically compatible with the packaged product, as established by appropriate compatibility assessments (e.g., stability studies).

Considering the fourth bullet point, ⟨661.2⟩ notes that appropriate chemical testing includes performing extractables testing, leachables testing, and the relevant toxicological assessment of the extractables and/or leachables results. In addition to being the basis for toxicological safety assessments, information about a packaging system's extractables can be used in several ways to optimize finished product testing for leachables. The potential quality and/or safety or impact of extractables may facilitate identification of leachables that might adversely affect product quality. Such leachables of potential concern would necessarily be among the targeted analytes in testing of a final pharmaceutical product within its packaging system. The targeting of specific leachables, as opposed to the screening of pharmaceutical products for unspecified leachables, has significant analytical benefits, including the ability to develop, validate, and utilize test procedures that are appropriately sensitive, specific, and accurate. Further, extractables (and their accumulation levels in extracts) can be used to forecast the levels of leachables in the finished product, depending on how well the extraction conditions mimic the pharmaceutical product's composition and actual conditions of clinical use. If the extraction conditions are such that they accelerate and modestly exaggerate the product's clinical use conditions, then the extractables and their levels in the extracts can be extrapolated to estimate the maximum levels of leachables in the finished product. Additionally, if such extractables are assessed for their safety or quality impact, the results of that assessment can also be extrapolated to, and deemed to be relevant for, the pharmaceutical product. Finally, if no adverse impact is found based on the extractables data, then no adverse impact can be inferred for the leachables in the packaged pharmaceutical product. Consistent with certain regulatory guidelines, leachables studies may not be required when extractables studies establish the maximum amount of individual leachables that may be present in the active substance/medicinal product and when such maximum levels have been demonstrated to be toxicologically safe. However, should a leachable study be deemed to be unnecessary, a justification should be provided.

APPLICABILITY AND APPLICATION OF ⟨661.1⟩

Applicability

1. The holder of the drug product application and drug product manufacturer [in the case of many over-the-counter products (OTCs), where there is no application] bear primary responsibility and accountability for ensuring the requirements of the chapter are met. The means by which the holder of the drug product application and drug product manufacturer obtains information to meet the requirement is at the discretion of the holder.
2. The testing required and specifications for materials of construction contained within ⟨661.1⟩ are relevant to and applicable for all drug dosage forms, as it is the universal expectation that packaging materials be constructed from well-characterized materials, regardless of the potential interaction between a dosage form. However, the use of risk-management principles and concepts to address the potential product safety risk associated with leachables (and extractables as potential leachables) is a cornerstone of global regulatory and industry thinking on this topic. Industrial scientists and regulators agree that the concepts and principles of risk management have a definite strategic role in terms of designing, implementing, and interpreting effective and efficient assessments of extractables and/or leachables. Oversimplifying somewhat, it is well-established that risk-management tools and principles can be used to define the nature and magnitude of assessment (including testing), where low-risk situations require reduced or alternate assessment (testing) versus high-risk situations. Thus, as noted in its *Tables 1* and *2*, ⟨661.1⟩ establishes biological reactivity and chemical tests that differ somewhat for low-risk dosage forms (such as oral and topical) versus high-risk dosage forms (such as inhalation and injections).
   Moreover, an essential principle reflected in ⟨661.2⟩ is that packaging systems be tested for extractables and that the approach be consistent with the nature of the interaction between the drug product and its packaging. This includes consideration of the drug product contact condition (e.g., liquid vs dry) and the potential interaction between the dosage form and its packaging system. By referencing ⟨1663⟩ for extractables testing, ⟨661.2⟩ provides the means by which extractables studies relevant for specific dosage forms can be designed, implemented, and interpreted. By allowing for study designs that reflect the nature and clinical use of various dosage forms, ⟨661.2⟩ supports and uses risk-based strategies and assessments.
3. The outcome of ⟨661.1⟩ testing is that the tested construction material has been well-characterized. Characterization data generated during ⟨661.1⟩ testing can be used to support decisions on the proper use of the tested material. However, the characterization data does not specifically or universally qualify the material for use in packaging systems, as the material's use can vary depending on the packaging applications. It is the responsibility of the developer or user of the tested material to decide if the material is appropriate for their intended application. Thus, it is the developer's or user's expert review of the ⟨661.1⟩ test results, coupled with additional information as necessary and appropriate, that establishes whether a well-characterized material is suitable for use in a specific application.
   Alternatively, the outcome of testing plastic packaging systems via ⟨661.2⟩ is an assessment of the probable safety impact of that system on the packaged drug product. This assessment is based on the biological reactivity testing, the physiocochemical testing, and the extractable/leachables testing that are required by ⟨661.2⟩. Thus, a packaging system that has been tested per ⟨661.2⟩ and which meets the specifications contained within ⟨661.2⟩, including a toxicological safety assessment of the extractables and/or leachables data, is qualified for use consistent with the conditions under which it was tested, subject to review by the appropriate regulatory authority.

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4. There are two means for demonstrating a material of construction has met the requirements of ⟨661.1⟩. The first means is direct testing and meeting the requirements in ⟨661.1⟩. The second means is the use of the material with a currently approved finished drug product.
5. Application of ⟨661.1⟩ and ⟨661.2⟩ to materials of construction or systems other than packaging systems for finished drug products is beyond the scope of these chapters, but the concepts and principles of these chapters may be applicable and relevant to other systems (and their materials for construction) such as medical devices for drug product administration, manufacturing systems for pharmaceutical products, and packaging/storage systems for drug substances. It is the expectation that future compendial chapters will be developed to address these other pharmaceutically important systems.
6. The scope of ⟨661.1⟩ is materials of construction and of ⟨661.2⟩ is packaging systems. A third type of test article, components, is not directly considered in the *Scope* of either chapter. In this context, a component is defined as an individual part of a packaging system and is constructed from one or more materials of construction. Thus, a plastic bag consisting of a laminated film is considered to be a component of the packaging system that includes the bag. Since a component is constructed from materials and is part of a system, if component testing is deemed to be necessary, the relevant testing and specifications for the component are contained within ⟨661.2⟩. The provisions in ⟨661.2⟩ for packaging systems must be met for components whose testing has been deemed to be necessary. The component must be constructed from materials that meet the requirements of ⟨661.1⟩ and the component must be tested by the methods, and meet the specifications, contained in ⟨661.2⟩.
7. Testing of materials of construction via ⟨661.1⟩ is predicated on the circumstance that the material will most likely interact with the packaged drug product when the material is used in a packaging system. It is not necessary for a material used in a packaging system to be well-characterized if there is little or no chance of the material and the packaged drug product interacting. Under these conditions the materials of construction would be considered non-interacting and would be exempt from ⟨661.1⟩ testing. The designation of a material of construction as "non-interacting" must be accepted by the appropriate regulatory authority.

Although it is beyond the scope of ⟨661.1⟩ to establish the means by which a material of construction is established as "non-interacting", it is relevant to differentiate between the potentially similar terms "no direct contact" and "non-interacting", where the term "no direct contact" means that the material and the packaged drug product do not come into direct physical contact under the clinical conditions of use. Although it may well be the case that in a specific application a "no direct contact" material of construction is also a "non-interacting" material of construction, it may also be the case that "no direct contact" does not insure "non-interacting", especially when the conditions of contact include long durations and/or substantially elevated temperatures.

To explain the concepts of "no direct contact" and "non-interacting", consider the following example. An aqueous drug product is packaged in a flexible plastic container. The flexible container is further placed in a foil overpouch. The overpouched product is terminally sterilized. An adhesive label is applied to the outside of the foil overpouch after the product unit has been cooled after terminal sterilization.

In this case, both the foil overpouch and the label are "no direct contact", as there is at least one physical barrier (the primary container) between the packaged drug product and these two items. However, if the flexible plastic primary container is permeable, the foil overpouch can be considered to be a "potentially interacting" component, as substances from the overpouch could migrate through the primary packaging, especially under the high-temperature conditions of terminal sterilization. On the other hand, the label is a "non-interacting" component because (1) the foil overpouch is impermeable and (2) the label is applied after the thermal stress associated with terminal sterilization.

Thus, the difference between a "potentially interacting" and "non-interacting" "no direct contact" component is the permeability of the barrier that separates the "no direct contact" components from the drug product. If the barrier is incomplete, then the component (and its materials of construction) is "potentially interacting" and the materials must be tested per ⟨661.1⟩. If the barrier is complete, then the component (and its materials of construction) is "non-interacting" and the materials need not be tested per ⟨661.1⟩.

Change to read:

Application

1. There are two means of demonstrating that a material of construction has met the requirements of ⟨661.1⟩. The first means is to perform the testing contained within ⟨661.1⟩ and meet the specifications in ⟨661.1⟩. The second means is the use of a material in the packaging system of a currently approved finished drug product. Specifically, ⟨661.1⟩ states "individual plastic materials of construction are deemed to be well-characterized and appropriate for use if they are used in a packaging system that meets the requirements in ⟨661.2⟩ or if the packaging system has been deemed appropriate for pharmaceutical use by the appropriate regulatory authority". However, it is noted that such a conclusion is only valid for the specific packaging system meeting the requirements of ⟨661.2⟩ and cannot be extended to other packaging systems using the same material (or materials) of construction. If the same material of construction is used in another packaging system, then its suitability for use in that packaging system must be established.
2. The outcome of ⟨661.1⟩ testing is that the tested material of construction has been well-characterized. Characterization data generated during ⟨661.1⟩ testing can be used to support decisions on the proper use of the tested material. However, the characterization data do not specifically or universally qualify the material for use in packaging systems, because the material's use can vary depending on the packaging applications. Alternately, the outcome of testing plastic packaging systems via ⟨661.2⟩ is an assessment of the probable safety impact of that system on the packaged drug product. This assessment is based on the biological reactivity testing, the physicochemical testing, and the extractables/leachables testing that are required by ⟨661.2⟩. Thus, a packaging system that has been tested per ⟨661.2⟩ and which meets the specifications contained within ⟨661.2⟩, including a toxicological safety assessment of the extractables and/or leachables

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data, is qualified for use consistent with the conditions under which it was tested, subject to approval by the appropriate regulatory authority.

3. The identification tests required in ⟨661.1⟩ serve the purpose of categorizing a material so that it is properly tested and evaluated against the appropriate specifications. The specifications for Identification are based on a comparison of the test result obtained for the test material versus the relevant Reference Standard. This comparison is based on the concept of substantial equivalence as opposed to exacting quantitative specifications. Establishing substantial equivalence requires that the test results and test material versus Reference Standard be judged to be equivalent. Although the individual specifications for the individual materials contained in ⟨661.1⟩ may include information that is relevant to establishing substantial equivalence, this information in and of itself is not deemed to be a specification. For example, although the infrared (IR) Identification specifications may include wavenumber targets, these targets are not specifications but rather serve the purpose of establishing the expected general characteristics of the IR spectra. An identification test is deemed to have been successfully completed if the analytical results obtained for the test article and the appropriate Reference Standard are substantially equivalent, and where all differences between the test results for the article and the Standard are explained by the nature, processing, and/or composition of the test article.
4. Establishing the potential safety impact of a material of construction cannot rely on a single testing strategy, as no single testing strategy is sufficient to identify all potential safety-impacting attributes of a material. Thus, the chemical testing ▲prescribed▲ (ERR 1-Jun-2018) in ⟨661.1⟩ is orthogonal: physicochemical tests provide a general overview of extracted substances; extractable metals tests address potential sources of elemental impurities; while plastic additives tests address potential organic extractables. It is also the case that chemical testing alone may not demonstrate all potential safety-impacting attributes. Thus, chemical testing is augmented by the orthogonal approach of establishing biological reactivity.
5. A well-characterized plastic material is tested for its extractable levels of all metals that are known components of the plastic material. They could originate from the starting materials used to manufacture the plastic material, reagents used in the manufacturing process (e.g., catalysts), and from additives present in the plastic materials. Such metals are termed "relevant metals". Additionally, materials are tested for metals that are specified in other compendial documents as being relevant for plastic materials. Lastly, materials are tested for metals that have been deemed to be elemental impurities that are applicable to all drug product dosage forms regardless of whether the source of the elemental impurities is intentionally or unintentionally added to the drug product, its ingredients, or its packaging system.
6. Extractable metals reporting thresholds contained within ⟨661.1⟩ are not to be construed as limits. Rather, the reporting thresholds establish the convention for reporting extractable metals results. In this regard, the *USP* specification for extractable levels is not that they be below a certain limit, but rather that they be reported as specified in ⟨661.1⟩.
7. It may be that not all of the relevant metals for a particular material of construction are specified in ⟨661.1⟩, and that some relevant metals become known by another means (for example, vendor certification). All relevant metals, regardless of their inclusion in ⟨661.1⟩, must be tested for. Procedures for relevant metals that are not specified in ⟨661.1⟩ must be established and should be consistent with the procedures used for metals that are specified in ⟨661.1⟩. Specifications must be established for relevant metals that are not specified in ⟨661.1⟩; such specifications should be consistent with the specifications established for metals that are specified in ⟨661.1⟩.
8. Extractable metals testing described in ⟨661.1⟩ is required for all materials of construction used in packaging systems, regardless of whether the material is specified in ⟨661.1⟩. Extractable metals test procedures for materials that are not specified in ⟨661.1⟩ must be established and should be consistent with the procedures used for materials that are specified in ⟨661.1⟩. Extractable metals specifications must be established for materials that are not specified in ⟨661.1⟩; such specifications should be consistent with the specifications established for materials that are specified in ⟨661.1⟩.
9. The listing of specific extractable metals in ⟨661.1⟩ is not meant to limit material sponsors or users who may seek to establish the level of extractable metals other than those specified in ⟨661.1⟩. This may be the case, as additional extractable metals may be applicable to certain dosage forms and as the analytical methods that may be applied to extractable metals analyses could routinely supply data for extracted metals other than those specified in ⟨661.1⟩. In cases where individual sponsors obtain test results for extractable metals other than those specified in ⟨661.1⟩, it is expected that such additional extractable metals would be reported in the manner specified in ⟨661.1⟩ for those extractable metals that are specified in ⟨661.1⟩.
10. The plastic additives testing described in ⟨661.1⟩ is required for all materials of construction used in packaging systems, regardless of whether the material is specified in ⟨661.1⟩. Procedures for materials that are not specified in ⟨661.1⟩ must be established and should be consistent with the procedures used for materials that are specified in ⟨661.1⟩. Specifications must be established for materials that are not specified in ⟨661.1⟩; such specifications should be consistent with the specifications established for materials that are specified in ⟨661.1⟩.
11. It is the responsibility of the user of materials that are not currently listed in ⟨661.1⟩ to: (a) develop those tests methods and specifications that are required per the points noted previously; (b) justify those test methods and specifications, specifically considering their consistency with test methods and specifications that exist for materials that are currently listed in ⟨661.1⟩; and (c) possess the test results obtained when the material is tested, in accordance with the tests outlined in the points noted previously.
12. Additionally, it is possible that materials specified in the chapter may contain additives that are not addressed in ⟨661.1⟩. These materials must be tested for such additives. Procedures for additives that are not specified in ⟨661.1⟩ must be established and should be consistent with the procedures used for materials that are specified in ⟨661.1⟩. Specifications must be established for additives that are not specified in ⟨661.1⟩; such specifications should be consistent with the specifications established for materials that are specified in ⟨661.1⟩.
13. The sole purpose of the tests for plastic additives is to establish which additives are present and to ensure that the levels of these additives are known. This information is relevant because additives are typically a source of extractables and leachables.

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14. The recommendation of specific tests, test methods, and test parameters in ⟨661.1⟩ does not preclude the use of other suitable methods, procedures, or parameters, but the conditions presented in ⟨661.1⟩ take precedence for official purposes. Alternative test methods and conditions must be demonstrated to be suitable by means of appropriate and sufficient validation data. Important aspects of alternative methods include the completeness of the extraction process and the specificity, sensitivity, and applicability of the analytical test methods. Extraction methods employed must have a demonstrated ability to quantitatively transfer additives from the material to the extracting medium and must do so without modifying the chemical nature of the additive unless such modification is an integral part of the test methodology. Test methods employed must have equivalent ability compared with the test methods contained in ⟨661.1⟩ to produce a clear and unambiguous identification of all relevant additives at levels at least as low as the levels specified in ⟨661.1⟩.
15. Point 14 notwithstanding, the substitution of alternate tests for those that are required by ⟨661.1⟩ is not appropriate. Thus, for example, substitution of an oxidizable substance test for the *Total Organic Carbon* test specified in *Physiochemical Tests* is not appropriate. Additionally, substitutions for specifications that exist in ⟨661.1⟩ are not allowed unless justified and are subject to approval by an appropriate regulatory authority.
16. In at least two places (*Extractable Metals* and *Plastic Additives*), ⟨661.1⟩ requires that materials be tested for all relevant analytes. Clearly, an analyte will be present in a material if it is intentionally or knowingly added to the material during its production or if testing of the material has revealed the analyte's presence. While test methods included in ⟨661.1⟩ may be of sufficiently broad scope to detect all relevant metals or additives, this is not always the case and one cannot rely on the methods to reveal all relevant analytes. It may be the case that the material's vendor has knowledge that may be unavailable to the material's user, which is germane to establishing relevant analytes. Thus, it is reasonable to expect that material vendors and users work together to produce a complete and robust list of relevant analytes. It is particularly important that a material's vendor inform the material's user when it is clear to the vendor that the user has missed a relevant analyte in the user's testing.

It is reasonable to anticipate that there may be some information that the vendor is not in a position to share with a material's user. Nevertheless, it is in the interest of both the vendor and the user that a material be well-characterized and that the characterization include all relevant analytes. Thus, it is strongly recommended that the vendor and the user find a means of establishing all relevant analytes. For example, consider the case of extractable metals. While it may be the case that the material's vendor would decline to share detailed information about the use of a zinc-containing reagent in the preparation of a material, it is adequate for the purpose of material characterization for the vendor to communicate that zinc should be a targeted analyte.

Change to read:

Description of Polymers Contained in ⟨661.1⟩

POLYETHYLENES

High- and low-density polyethylene are long-chain ethylene-based polymers synthesized under controlled conditions of heat and pressure with the aid of catalysts from NLT 85.0% ethylene and NLT 95.0% total olefins. Other olefin ingredients that are most frequently used are butene, hexene, and propylene. Low-density ▲polyethylene▲ (ERR 1-Jun-2018) (LDPE) contains many long-chain branches along the polymer backbone, preventing the alignment and packing of the chains and thus forming a low-density material. Linear low-density polyethylene (LLDPE) contains several short chains along the polymer backbone that prevent the alignment and packing of the polymer chains, thus creating a poor crystalline material. High-density polyethylene (HDPE) contains relatively few side chains, allowing the polymer backbone to align and pack together, thus forming a crystalline, high-density plastic. High-, low-, and linear low-density polyethylene all have an IR absorption spectrum that is distinctive for polyethylene, and each possesses characteristic thermal properties. High-density polyethylene has a density between 0.941 and 0.965 g/cm^3. Low-density polyethylene has a density between 0.850 and 0.940 g/cm^3. Additives are added to the polymer in order to optimize its chemical, physical, and mechanical properties, thereby rendering it suitable for its intended use. These additives may include nucleating agents, clarifying agents, antioxidants, colorants, lubricants, antiblocking agents, and others. These additives typically are present individually in the polyethylene at levels of 0.01 to 0.3 weight %, and the total levels of the antioxidants typically are less than 0.3%. Other additives, specifically amides and stearates, typically are present in polyethylenes individually at levels of 0.5 weight % or less. Polyethylene materials that provide light protection can contain as much as 4% by weight titanium oxide.

POLYPROPYLENE

Propylene polymers are long-chain polymers synthesized from propylene or other olefins, for example, ethylene or butene, under controlled conditions of heat and pressure with the aid of catalysts. A certain number of additives are added to the polymer in order to optimize its chemical, physical, and mechanical properties, thereby rendering it suitable for its intended use. These additives may include nucleating agents, clarifying agents, antioxidants, colorants, lubricants, antiblocking agents, and others. These additives typically are present individually in the polypropylene at levels of 0.01 to 0.3 weight %, and the total levels of the antioxidants typically are less than ▲0.3%. Polypropylene▲ (ERR 1-Jun-2018) that provides light protection can contain as much as 4% by weight titanium dioxide.

CYCLIC OLEFINS

Cyclic olefin copolymers are manufactured by the copolymerization of a cyclic olefin (e.g., cyclopentene, norbornene) with an olefin such as ethylene or propylene. The reaction of polymerizing a cycloolefin resulting in a polymer is known as ring

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opening polymerization (ROMP) and is facilitated via Ziegler-Natta catalysts. Cyclic olefin polymer resins are commonly supplied in pellet form and are suited for standard polymer processing techniques such as extrusion, injection molding, injection blow molding, compression molding, thermoforming, and others. As they are amorphous, and given their high purity, moisture barrier, clarity, and sterilization compatibility, cyclic olefins are an excellent alternative to glass in a wide range of medical products, including packaging. Cyclic olefins exhibit good chemical resistance and are generally considered to be of high purity with low levels of extractables. Nevertheless, cyclic olefin copolymers may contain residual processing aids, colorants, and antioxidants.

POLYETHYLENE TEREPHTHALATE AND POLYETHYLENE TEREPHTHALATE G

Polyethylene terephthalate (PET) polymers are long-chain crystalline polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid. PET copolymer resins are prepared in a similar way except that they may also contain a small amount of either isophthalic acid (NMT 3 mole %) or ▲1,4-cyclohexanedimethanol▲ (ERR 1-Jun-2018) (NMT 5 mole %). Polymerization is conducted with the aid of catalysts and stabilizers. PET polymers may contain silica or silicates (NMT 0.5% by weight) and may contain colorants.

PLASTICIZED POLY(VINYL CHLORIDE)

Poly(vinyl chloride) (PVC) polymers are long-chain vinyl chloride polymers synthesized from vinyl chloride monomers via free radical polymerization. Various additives are compounded into PVC to provide the materials with properties that render it suitable for its intended use. These additives may include heat stabilizers, primary and secondary plasticizers, stabilizers, impact modifiers, lubricants, pigments, and others. These additives typically are present individually in the PVC at levels ranging from 0.1 to 45 weight %.

APPLICABILITY AND APPLICATION OF ⟨661.2⟩

Applicability

1. The holder of the drug product application and drug product manufacturer (in the case of many OTCs, where there is no application) bear primary responsibility and accountability for ensuring the requirements of the chapter are met. The means by which the holder of the drug product application and drug product manufacturer obtain information to meet the requirement is at the discretion of the holder.
2. Chapter ⟨661.2⟩ deals solely with packaging systems. Components of packaging systems can be tested per ⟨661.2⟩ at the discretion of the holder of the drug product application and as approved by regulatory authority. Materials of construction are not tested per ⟨661.2⟩.

Application

1. Chemical characterization of either extracts of packaging systems (extractables) or of packaged drug products (leachables), followed by toxicological safety evaluation, is universally recognized as a necessary and appropriate means of establishing the safety impact between packaging systems and their contents. Thus, ⟨661.2⟩ requires that all packaging systems be demonstrated to be safe by performing a chemical assessment. However, ⟨661.2⟩ does not specify the details of the chemical assessment process, either in terms of the test methods or the specifications. Rather, ⟨661.2⟩ references the relevant informational chapters (⟨1663⟩ for extractables and ⟨1664⟩ for leachables) thereby providing users of ⟨661.2⟩ with a means for designing and implementing an effective, efficient, risk-based, and more or less customized extractables or leachables assessments that comply with regulatory requirements.
2. Chapter ⟨661.2⟩ provides holders of packaging system or drug product applications and/or packaged drug products manufacturers with the flexibility to operate within the context of their own specific situation and their own specific risk-management philosophy. The trade-off for having such flexibility is that it is the responsibility of the holders and manufacturers to justify their test methods and specifications. It is proper and appropriate that the justification exists and that it be judged (and approved) on the basis of its individual scientific and risk-management merits.
3. Leachables whose chemical formula includes transition metals, metalloids, other metals, and lanthanides and actinides are elemental impurities. To the extent that extractables mirror leachables, extractables can be construed to be potential elemental impurities. *Elemental Impurities—Limits* ⟨232⟩ contains specifications for elemental impurities in drug products. In some manner, these specifications are relevant to packaging systems, as leachables of the appropriate composition represent a certain proportion of a drug product's elemental impurity burden. However, if the proportion is not known, then ⟨232⟩ specifications for drug products cannot be directly translated to specifications for leachables which themselves are elemental impurities. Thus, ⟨661.2⟩ requires that leachables that are elemental impurities be appropriately assessed toxicologically for their potential safety impact and correctly notes the existence of ⟨232⟩. However, ⟨661.2⟩ does not specifically attempt to use the product specifications in ⟨232⟩ to set leachables specifications as the means to establish safety impact.

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⟨1663⟩ ASSESSMENT OF EXTRACTABLES ASSOCIATED WITH PHARMACEUTICAL PACKAGING/DELIVERY SYSTEMS

PURPOSE

This general information chapter presents a framework for the design, justification, and execution of an extractables assessment for pharmaceutical packaging and delivery systems. The chapter establishes critical dimensions of an extractables assessment and discusses practical and technical aspects of each dimension. Although intended to be helpful and generally applicable, the chapter is for informational purposes and does not establish specific extraction conditions, analytical procedures, or mandatory extractables specifications and acceptance criteria for particular packaging and delivery systems or dosage forms; nor does it delineate every situation in which an extractables assessment is required. It is not possible for a general discussion of extractables to anticipate and cover all situations where an extractables assessment might be required. Designing an individual extractables assessment is a process that balances sound science, prudent resource allocation, and effective risk management. Achieving this balance is the responsibility and obligation of the drug product manufacturer and assumes due consideration of all applicable legal and regulatory requirements. The principles and best demonstrated practices outlined in this general chapter represent a consensus interpretation of sound science and can therefore be applied to any situation in which an extractables assessment is required for pharmaceutical application.

KEY TERMS

This general chapter uses the following key terms listed below (*1*, *2*; also see *Packaging and Storage Requirements* ⟨659⟩). Note that the terms *Packaging System*, *Packaging Component*, *Primary Packaging Component*, *Secondary Packaging Component*, and *Materials of Construction* are also defined in ⟨659⟩, and the definitions below are intended for clarification purposes within the context of this chapter and are not intended to supersede those provided in ⟨659⟩.

Packaging System (also referred to as a container–closure system): The sum of packaging components and materials that together contain and protect the product. This includes primary, secondary, and tertiary packaging components.

Delivery System: The sum of components and materials that are used to transport a drug product from its packaging to the point of administration into the patient. For example, an administration set is a delivery system that is used to transfer liquid drug products from their plastic packaging system to the site of administration to the patient. It is noted that in some cases, the packaging system itself may perform the delivery function.

Container: A receptacle that holds an intermediate compound, active pharmaceutical ingredient, excipient, or dosage form and is in direct contact with the product.

Closure: A material that seals an otherwise open space on a container and provides protection for the contents. It also provides access to the contents of the container.

Packaging Component is any single part of the package or container–closure system including the container (e.g., ampuls, prefilled syringes, vials, bottles); closures (e.g., screw caps, stoppers); ferrules and overseals; closure liners; inner seals; administration ports; overwraps; administration accessories; labels; cardboard boxes; and shrink wrap.

Primary Packaging Component is a packaging component that is in direct contact or may become in direct contact with the product (e.g., IV bag).

Secondary Packaging Component is a packaging component that is in direct contact with a primary packaging component and may provide additional protection for the product (e.g., overpouch or dustcover for an IV bag).

Tertiary Packaging is a packaging component that is in direct contact with a secondary packaging component and may provide additional protection of the product during transportation and/or storage (e.g., shipping carton for an overpouched IV bag).

Ancillary Component is a component or entity that may come into contact with a tertiary packaging component during the distribution, storage, and transportation of the packaged product (e.g., pallets, skids, shrink wrap).

Packaging Materials of Construction are substances used to manufacture packaging components. These are also referred to as *Raw Materials*.

Extractables are organic and inorganic chemical entities that are released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction and into an extraction solvent under laboratory conditions. Depending on the specific purpose of the extraction study (discussed below), these laboratory conditions (e.g., solvent, temperature, stoichiometry, etc.) may accelerate or exaggerate the normal conditions of storage and use for a packaged dosage form. Extractables themselves, or substances derived from extractables, have the potential to leach into a drug product under normal conditions of storage and use and thus become leachables.

Leachables are foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. Because leachables are derived from the packaging or delivery system, they are not related to either the drug product itself or its vehicle and ingredients. Leachables are present in a packaged drug product because of the direct action of the drug product on the source of the leachable. Thus leachables are typically derived from primary and secondary packaging, as the primary and secondary packaging can serve as a barrier between the packaged drug product and other potential sources of foreign chemical entities (such as tertiary packaging and ancillary components). In certain circumstances, packaging may directly contact the patient under typical clinical conditions of use (for example, the mouthpiece of a metered dose inhaler). As a result of this contact, patients may be exposed to leachables from the packaging without the action of the drug product. Leachables are typically a subset of extractables or are derived from extractables.

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Migrants are also foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. However, migrants are differentiated from leachables by the circumstance that migrants accumulate in the packaged drug product after the migrant has crossed a physical barrier, such as that provided by primary and secondary packaging. Because migrants cross a physical barrier, they are not present in the packaged drug product due to direct action of the drug product on the source of the migrant because the barrier prevents such direct action. Thus migrants are derived from secondary and tertiary packaging and ancillary components. Regardless of whether a substance is a leachable or a migrant, it is still a foreign substance in the packaged drug product and thus it must be impact assessed in the same manner. However, as the means by which a leachable and a migrant become entrained in a packaged drug product may be different, extractables studies meant to address leachables may be designed and implemented differently than extractables studies meant to address migrants.

Extraction Studies are the overall laboratory processes required in order to create extractables profile(s) of particular pharmaceutical packaging/delivery systems, packaging components, or materials of construction. Extraction studies are also referred to as *Controlled Extraction Studies*.

Characterization is the discovery, identification, and quantitation of each individual organic and inorganic chemical entity present in an extract above a specified level or threshold. Such thresholds can be based on patient safety considerations, materials considerations, the capabilities of analytical technology, etc.

Scouting is the process of acquiring general chemical information that provides insight into the nature and magnitude of extractables.

Discovery is the process of searching for, and ultimately finding, individual organic and inorganic chemical entities present in an extract.

Identification is the process of assigning a molecular structure to an organic extractable, or assigning constituent elements in the case of an inorganic extractable.

Quantitation is the process of measuring the level, or concentration, of an individual organic or inorganic chemical entity contained in an extract.

Extractables Profiles are qualitative and/or quantitative analytical representations of the extractables content of a particular extracting medium and set of laboratory extraction conditions.

Leachables–Extractables Correlations are established when observed drug product leachables are linked both *qualitatively* and *quantitatively* to extractables from associated packaging/delivery systems, packaging components, or materials of construction.

Safety Concern Threshold (SCT) is the threshold below which a leachable has a dose so low that it presents negligible safety concerns from carcinogenic and noncarcinogenic toxic effects.

Analytical Evaluation Threshold (AET) is the threshold at or above which a leachable should be characterized and reported for toxicological assessment. The AET can be mathematically derived from the SCT (or other threshold concepts) based on factors that include the dosing parameters of the drug product. When an extraction study is performed for the purpose of estimating the accumulation levels of leachables, the AET may be applicable to extractables as well as leachables. The concept of the AET is discussed in greater detail in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

As noted, additional terminology and associated definitions are available (*1*, *2*, and ⟨659⟩).

SCOPE

The scientific principles and best-practices described in this general chapter are intended to apply to any extractables assessment or extraction study of pharmaceutical packaging/delivery systems, packaging components, or materials of construction; the results of which are intended for establishing extractables profiles. Extractables profiles can be used in a variety of pharmaceutical development and manufacturing applications, including the characterization, selection, and qualification of materials of construction, packaging components or packaging/delivery systems; establishing leachables–extractables correlations; and/or simulating worst-case drug product leachables profiles. When appropriate, extractables profiles can also be used in establishing leachables–extractables correlations as described in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩. These scientific principles and best practices can also be applied to materials of construction and functional components of equipment used to manufacture drug substances and drug products; e.g., filters, tubing, and tanks. In addition, these principles and best practices can be applied to materials of construction for the medical device component of a combination product (*2*), with appropriate consideration of the guidances and regulations that apply specifically to medical devices. These scientific principles and best practices apply to all organizations and individuals involved in the manufacture of drug substances, drug products, and in their stability studies, including but not limited to:

- Manufacturers of drug substances and drug products for human and veterinary use where manufacturing may involve operations at the applicant holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Manufacturers of combination products
- Packaging operations by the manufacturer or a designated contractor for the application holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer

Manufacturers and fabricators of pharmaceutical packaging/delivery systems, packaging components, and materials of construction may also apply these scientific principles and best-practices as appropriate.

BACKGROUND INFORMATION

During the course of manufacturing, packaging, storage, distribution, and administration; dosage forms and their constituents can contact components and materials of construction of manufacturing and packaging equipment, and primary

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and secondary packaging components and systems. Such contact may result in interactions between the dosage form and these components and materials. One such interaction is the migration or leaching of substances from any of these components and materials into the dosage form with subsequent delivery to the patient during drug administration. Patients also can be directly exposed to substances via direct contact with the packaging/delivery system during drug administration. Leachables, which can include both organic and inorganic chemical entities with wide chemical diversity, are of concern due to their potential safety risk to patients and potential compatibility risks for the drug product (e.g., drug substance interaction/degradation, pH change, appearance change, particle formation, protein aggregation/structure change, etc.). In order to assess these risks and manage the potential issues posed by leachables, it is necessary to know the identities and the levels to which leachables will accumulate in the finished drug product over its shelf-life. These two pieces of information can be used to establish the magnitude of patient exposure (dose) and therefore the safety risk posed by an individual leachable, as well as the likelihood of any compatibility issues involving the drug product.

Regulatory guidelines and various best-practice recommendations state that assessment of the potential impact of contact between a component or material and a final dosage form involves evaluating the final dosage form with respect to leachables. This assessment can include a migration or leachables study whose purpose is to discover, identify, and quantitate leachables that have migrated from the contacted system, components, or materials and accumulated in the dosage form. Alternatively, this assessment may involve performing a simulation extraction study, when use of such a study in lieu of a migration study can be justified. There are many science-based and practical reasons why such a leachables assessment typically does not stand alone as the single means of assessment. Since the pharmaceutical packaging/delivery system is the primary source of potential leachables, it is generally appropriate that any leachables assessment be preceded by an extractables assessment performed on the packaging/delivery system, its primary and certain critical secondary packaging components that are noncontacting but potentially interacting, and/or packaging and delivery system materials of construction; consistent with regulatory guidelines and best-practice recommendations. Such an extractables assessment can also be performed on particular components and/or materials of construction of manufacturing and packaging equipment, as well as certain tertiary packaging components, that are deemed of high leaching potential or have been implicated in an identified leachables problem with a particular drug product.

Extractables assessments can be used to:

- Characterize packaging/delivery systems, packaging components, combination product medical device components, manufacturing components, and their various materials of construction
- Facilitate the timely development of safe and effective dosage form packaging/delivery systems, manufacturing systems and processes by assisting in the selection of components and materials of construction
- Understand the effects of various manufacturing processes (e.g., sterilization) on packaging components and their potential leachables
- Establish the worst-case potential leachables profile in a manner which facilitates leachables studies, the development of leachables specifications and acceptance criteria (should these be required), and the safety evaluation/qualification of potential and actual leachables
- Establish the worst-case potential leachables profile in a manner which facilitates the safety evaluation/qualification of probable leachables when it is not scientifically possible to determine actual leachables
- Facilitate the assessment of patient exposure to chemical entities resulting from direct contact between a patient's body tissue(s) (e.g., mouth, nasal mucosa) and a packaging or combination product medical device component (e.g., a metered dose inhaler's plastic actuator/mouthpiece)
- Facilitate the establishment of qualitative and quantitative leachables–extractables correlations
- Facilitate the development of extractables specifications and acceptance criteria (if these are required) for packaging components, combination product medical device components, and materials of construction
- Facilitate investigations into the origin(s) of identified leachables whose presence causes quality and/or safety issues (such as out-of-specification results) for a marketed product

In these ways, extractables assessments can support Quality by Design (QbD) principles for the development and manufacture of pharmaceutical packaging/delivery systems and drug products. Note that although characterization of packaging/delivery systems and materials is a goal of many extractables assessments, regulatory guidances and best-practice recommendations clearly stress that extractables assessments also serve as investigations into potential leachables (*1–5*).

As stated previously, it is not a goal of this chapter to identify each case in which an extractables assessment is required for packaging/delivery systems, individual packaging components, or materials of construction for any particular type of dosage form. This is the responsibility of the Holder of the NDA (applicant holder), and assumes appropriate consideration of applicable regulatory guidance documents. Rather, this chapter addresses the question "If an extractables assessment is required, what are the scientific principles and best demonstrated practices under which it should be accomplished?"

As performing an extractables assessment involves the processes of discovery and identification, an extractables assessment can be facilitated by knowledge about the test article, especially its composition. Thus it is strongly recommended that the test article assessor and the test article vendor collaborate in such a way that the test article assessor has access to critical information which will aid in the design and implementation of an effective and efficient extraction study. Additionally, it is noted that characterization of the test article per *Plastic Materials of Construction* ⟨661.1⟩ will provide information that is useful in the design and implementation of extraction studies.

Achieving the objectives of an extractables assessment requires performance of an extraction study in order to create extractables profiles. An extraction study has two critical dimensions: laboratory generation of the extract (extraction) and testing the extract (characterization).

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GENERATING THE EXTRACT

General Concepts and Critical Experimental Design Parameters

Extractables are derived from a variety of sources and exhibit extensive chemical diversity. Primary sources of extractables include:

- Chemical additives in individual elastomeric/polymeric packaging components and raw materials, including impurities in these additives
- Chemical entities and additives that are present in packaging components composed of glass and metals
- Entities related to the dissolution of the packaging component itself (e.g., iron extracted from a stainless steel material, silicon extracted from glass)
- Monomers and higher molecular weight oligomers derived from incomplete polymerization
- Migrants from secondary and tertiary packaging components, such as inks, label adhesives, and volatiles from cardboard shipping containers, plastic storage bags, and other shipping aids such as wooden pallets
- Surface residues, such as heavy oils and degreasing agents on metal canisters and containers
- Chemical substances on the surfaces of component fabrication machinery or other drug product manufacturing systems, such as mold release agents, and antistatic and antislip agents
- Chemical additives, monomers/oligomers, impurities, etc., in various parts of component fabrication machinery or other drug product manufacturing systems

As noted above, the chemical diversity of extractables is significant. For example, the chemical additive category *antioxidants* includes hindered phenols, secondary aromatic amines, hindered amines, organosulfur compounds, organophosphorus compounds, and other chemical classes.

Extraction is a process of treating a material with a solvent to remove soluble substances. Extraction is a complex process influenced by time, temperature, surface area to volume ratio (i.e., stoichiometry), extraction medium, and the phase equilibrium of the material (*3*). There are two reasons why an extraction study is the necessary and appropriate means of accomplishing the various objectives of an extractables assessment. First, there is no other viable analytical alternative. Characterizing a material for potential leachables in its natural solid state is a goal of modern analytical chemistry rather than an accomplishment. Second, even if a direct characterization could be accomplished, it would at best only establish the identities and levels of chemical entities present in the material, and not assess the leaching characteristics of these chemical entities. A compositional assessment does not take into account any chemical reactions that can alter the molecular structures of potential leachables over the dosage form's lifecycle. For example, in the case of phenolic antioxidants that are leached by an aqueous drug product, hydrolysis and oxidation products can accumulate in the drug product. The only viable means for producing data related to leaching is to use a process such as laboratory extraction that is mechanistically similar to leaching.

The design of an extraction study is dictated by the purpose of the extractables assessment and the question(s) being asked, as well as the available information regarding the chemical composition of the test article(s) to be extracted. Extraction studies can be designed to answer questions such as:

- What are the chemical additives in a particular packaging component or material of construction?
- What are the maximum accumulations of chemical additives from a particular packaging component into the dosage form?
- What are the likely contents of an end-of-shelf-life drug product leachables profile?

Addressing each of these questions requires a particular set of parameters, such as extraction time, extraction temperature, extracting solvent, extraction technique, sample surface area to extracting solvent volume ratio, etc. Clearly, the intent of the extractables assessment must be established before the study design is finalized. For example, if the purpose of the extraction study is to simulate a worst-case leachables profile, then the study can be termed a simulation study that should produce an extract that:

- Contains all the substances (extractables) that could leach into the final product at levels considered potentially significant
- Contains these extractables at concentrations that are greater than or equal to the maximum concentration that these chemical entities will accumulate in the drug product as leachables at any time during the shelf-life
- Is generated more efficiently and in less time than that required for a drug product leachables study
- Is amenable to chemical analysis

The concept of a simulation study is addressed in greater detail in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩. The means by which an extraction process is accomplished are reflected in the juxtaposition of several experimental parameters including:

- The chemical nature of the extracting media
- The time duration of the extraction process
- The temperature and pressure at which the extraction is performed
- The stoichiometry of the extraction process (extracted surface area per unit volume of extracting solution)
- The mechanism or process by which the extraction is accomplished

Extraction processes have been described as "accelerated", "aggressive", "exhaustive", "vigorous", "harsh", and so on, and for medical device studies certain of these terms have been defined. In general, extraction processes should allow completion in a reasonable time frame but should not be so aggressive that they alter the qualitative and/or quantitative nature of the resulting extractables profile. The most aggressive extraction conditions are reserved for the quantitative determination of chemical additive contents in components and materials. Because such studies are intended to quantitate specific known chemical additives and not to simulate a drug product leachables profile, it is acceptable to use extraction conditions which

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disrupt or dissolve the component or material being extracted, and thus to alter the resulting extractables profile, while recovering the target additive(s) without loss or chemical decomposition.

Chemical Nature of the Extracting Medium

Of all the parameters involved in generating the extract, the extracting medium is the most critical because it is the extracting medium that accomplishes the extraction, and all other parameters merely facilitate the extraction. Establishing and justifying the extracting medium (or media) is both straightforward *strategically* and complex *tactically*. *Strategically*, if the purpose of a particular extraction study is, for example, to simulate a worst-case leachables profile, then the ideal situation is for the extracting solvent to have a similar or greater propensity to extract substances as the formulation, thus obtaining a similar qualitative and quantitative extractables profile. This is clearly stated in regulatory guidances and best practice recommendations (*1*, *4*, *5*). Therefore, the most logical *tactic* for this simulation study is to use the formulation itself as the extracting medium and in the absence of complicating factors, such an approach is recommended. However, in certain cases the use of the formulation as an extracting medium complicates extract characterization to such an extent that it is impractical. The various guidances and recommendations suggest that if the use of the drug product as the extracting solvent is not feasible, then the drug product vehicle, or placebo, could be used as an effective extracting medium. This recommendation is derived from the fact that the drug substance itself does not typically create the "leaching power" of a drug product but rather that it is the formulation's ingredients (drug product vehicle) that establish the drug product's ability to leach substances from a contacted material.

When circumstances require that an extraction study must be accomplished with a simulating solvent(s), it is necessary to establish and justify the composition of these solvents. In order to accomplish this objective, one must consider all the physicochemical characteristics of a formulation and/or simulating solvent that influence its "extracting power". In certain circumstances, the formulation is sufficiently simple that the critical characteristics can be readily delineated and simulated. For example, the extracting power of polar aqueous drug products consisting of soluble ingredients (such as an injectable with a drug substance, buffers, and diluent) is, for organic extractables, driven primarily by drug product pH. In such a circumstance, simulating the drug product pH with an analytically viable buffer system for the extraction study may be appropriate and justifiable. For inorganic extractables, utilization of a simulating solvent having similar metal-chelating properties as the drug product vehicle may also be appropriate and justifiable. It may also be the case that largely non-polar drug products can be readily simulated with analytically expedient organic solvents. For example, chlorofluorocarbon and hydrofluoroalkane propellants used in metered dose inhalers (MDIs) can be simulated with dichloromethane as an extracting solvent and isopropanol can be used to simulate ethanol, a common co-solvent in MDI formulations.

Many drug products are compositionally intermediate between the polar and nonpolar examples just discussed. Examples of such products include "aqueous" drug products that contain stabilizers, solubilizing agents, chelating agents and buffers, lipid-containing products, and biotechnology products containing proteins, peptides, and blood-derived products. Such products have a characteristic polarity which establishes their "extracting power". Thus, an appropriate simulating solvent will have a polarity that matches that of the drug product. Binary mixtures of miscible solvents (such as alcohol/water) have been utilized as simulating solvents for these types of drugs product.

It may be that a single simulating solvent cannot be established and justified for a specific drug product, or that the focus of the extractables assessment is a material or system that will be characterized for use with multiple, compositionally diverse drug products. In such circumstances, the drug product's ability to leach chemical entities from a packaging system can be established based on the use of multiple extracting solvents, each of which addresses one (or more) of the extracting "mechanisms" that are relevant to the drug product (or drug products) under investigation. The use of multiple solvents is consistent with industry-driven best-practice recommendations for drug products that have a relatively high risk of dosage form interaction with the packaging system and a relatively high safety risk relative to the route of administration (e.g., inhalation aerosols and solutions, injectables and injectable suspensions) (*1*). Therefore, the use of multiple solvents (or extracting media) with different polarities, pH, ionic strength, or extracting powers, is recommended for high risk dosage form packaging system components and materials requiring extraction studies in order to simulate a drug product leachables profiles (see *Table 1*). If the goal of an extractables assessment is materials characterization, then simulating a drug product vehicle is both unnecessary and undesirable since this goal requires qualitatively and quantitatively efficient extractions. Such extractions generally are only achieved with relatively powerful organic solvent systems capable of softening, swelling, or dissolving the material's polymer matrix and releasing quantitative levels of additives and other chemical entities.

Table 1. Possible Extracting Media Relative to Particular Packaging Components

Packaging Component	Possible Extracting Media[a]
MDI valve elastomer seal (MDI formulation contains 1,1,1,2-tetrafluoroethane and ethanol)	Nonaqueous solvents (e.g., Dichloromethane Isopropanol Hexane)[b]
Dry powder inhaler mouthpiece	Water (unbuffered) Isopropanol[c]
Small-volume parenteral vial rubber stopper (aqueous formulation buffered at pH 6.5)	Water (pH 5.2) Water (pH 9.5) Isopropanol:water (50:50)[d]

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Table 1. Possible Extracting Media Relative to Particular Packaging Components *(continued)*

Packaging Component	Possible Extracting Media[a]
Large-volume parenteral plastic bag (aqueous formulation buffered at pH 7.2)	Water (pH 5.2) Water (pH 9.5) Isopropanol:water (50:50)[d]

[a] The possibilities listed in *Table 1* are provided for example only and should not be interpreted as standard practice recommendations.
[b] These extraction media reflect the varying polarities of the organic solvents used in MDI formulations.
[c] These extraction media reflect both the hydrophilic and lipophilic character of human saliva and allow materials characterization.
[d] These extraction media reflect the chemical nature of the formulation. Using media whose pH range encompasses, and slightly exceeds, the pH limits of the product addresses the potential effect of pH on the extractables profile. The use of an aqueous mixture containing an organic solvent takes into account the possible presence of formulation additives such as solubilizing agents that can influence the leaching power of the formulation. The specific organic solvent used and its proportion in the extracting medium depends on the specific chemical nature of the formulation and on practical issues associated with testing the extract.

If the goal of an extractables assessment is materials characterization, then simulating a drug product vehicle is both unnecessary and undesirable since these goals require qualitatively and quantitatively efficient extractions. Such extractions generally are only achieved with relatively powerful organic solvent systems capable of softening, swelling, or dissolving the material's polymer matrix and releasing quantitative levels of additives and other chemical entities.

Extraction Time and Temperature

Extraction time and temperature are critical factors in the extraction process. Although the nature of the extraction solvent establishes the magnitude of the extraction (i.e., the amount of substances that can be extracted from a material at equilibrium), the combination of extraction time and temperature establishes the magnitude of the driving force and the degree to which equilibrium is actually achieved. In a simulating extraction study the purpose of elevated temperature is to increase the extraction rate, so that a short experimental time may simulate longer leaching times (*1*, *5*, and *6*).

Because extraction is a diffusion process, the relationship between the diffusion rate and temperature can be expressed empirically by the Arrhenius equation. The mathematics involved in a process that is driven by Arrhenius kinetics have been established in ASTM F1980-07 (2011) Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices (*7*), which may be a useful guide for establishing accelerated contact conditions. As with all such models, the proper use of this model requires an understanding of the model's basis and essential principles, assumptions, and limitations (*2*).

Extractables profiles obtained with a given extracting medium and extraction technique can and should be monitored for equilibrium or the attainment of asymptotic levels of extractables (see *Figure 1*).

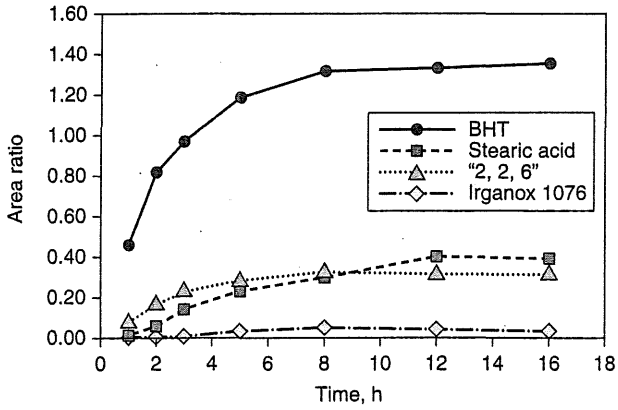

Figure 1. A graphical representation of an extraction that has attained equilibrium as indicated by the achievement of asymptotic levels of target individual extractables as a function of extraction time (i.e., GC/MS peak area ratios of target extractables relative to an internal standard plotted versus extraction time).

Extraction Stoichiometry

Extraction stoichiometry considers the physical mass and/or surface area of the test article relative to the volume of the extracting medium, and the actual physical state of the material when it is extracted. Extraction stoichiometry can be manipulated to facilitate production of a more concentrated extract. For example, consider the case of a rubber stopper for a vial that contains 5 mL of a liquid drug product. A more concentrated extract than the drug product (i.e., an extract that contains higher levels of extractables than the leachables level in the drug product) could be produced by extracting 20 stoppers in 200 mL of extracting solvent. Another aspect of extraction stoichiometry is the physical state of the test article. It is not uncommon that components or materials are cut, opened, ground, or otherwise altered in size or configuration prior to being extracted. For inhomogeneous or layered materials such as film laminates, the process of cutting or grinding prior to extraction may alter the extractables profile as it may provide a means for the extracting solvent to come into contact with (and thus more effectively extract) materials (layers) that are shielded from contact with the solution under normal conditions of use. One can argue that the use of such sized material further facilitates the extraction process, however it is possible for sizing to reveal extractables that might not appear as leachables. Nevertheless, some sizing of components or materials before extraction can

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be useful in certain situations and for certain purposes, including: (a) reducing sample-to-sample variability by the consistent preparation of ground homogeneous polymeric material; (b) reducing the sizes of large packaging components to allow use of standard laboratory glassware for extraction studies; (c) increasing the surface area of a packaging component or material test article (e.g., via extruding, pressing, or grinding) in order to increase extraction efficiency. In any event, careful consideration should be given to the effect of physical sizing of test articles on the extractables profile before such sizing methods are employed in extraction studies.

For extractables assessments involving components or materials whose chemical ingredients are known based on information from the supplier or fabricator, analysts can manipulate extraction stoichiometry based on the known levels of chemical additives and the known sensitivity of the analytical technique(s) that will be used to characterize the extract. For example, consider the formulation for a peroxide-cured ethylene-propylene-diene-monomer (i.e., EPDM) gasket from an MDI valve shown in *Table 2*.

Table 2. Ingredients in a Peroxide Cured Rubber Gasket Test Article that are Used in an MDI

Elastomer Ingredient	Amount (Nominal)
EPDM polymer	64.0%
Mineral fillers (may include stearic acid)	34.4%
Antioxidant 1: (butylated hydroxytoluene)	0.3%
Antioxidant 2: (2,2′-methylene-bis-[6-(1,1-dimethylethyl)-4-methyl] phenol)	0.3%
Peroxide curing agent	1.0%

Such information, when available from component and material suppliers, can be useful in designing an extraction study.

Analysts can also base the extraction stoichiometry on established safety thresholds for leachables. For example, an exposure of 0.15 µg/day total daily intake for an individual organic leachable has been proposed as an SCT for inhalation drug products, also termed orally inhaled and nasal drug products. Leachables present at or above the SCT, in an MDI for example, should be analytically and toxicologically evaluated, suggesting that extractables assessment also should be guided with the SCT in mind. The application of thresholds such as the SCT and AET to leachables assessments is discussed in greater detail in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

In summary, extraction stoichiometry (and thus the "sensitivity" of an extraction study) can be based on:

- The known chemical ingredients in a component or material
- Safety-based thresholds for drug product leachables
- The known or determined sensitivities of analytical instrumentation used for extract characterization

Mechanism of Extraction—Extraction Technique

An extraction can be accomplished in a variety of ways. It is necessary that the means of performing the extraction match the objectives of the extractables assessment. Common laboratory extraction techniques include:

- *Maceration (solvent soaking)*—in which the test article is allowed to soak for a period of time in an organic or aqueous extracting solvent at temperatures below the solvent's boiling point. Analysts can also fill packaging system units with extracting solvent and store them at relevant temperatures.
- *Reflux*—in which the test article is immersed in boiling solvent for a period of time.
- *Soxhlet*—in which the test article is placed in the "thimble" of a Soxhlet extraction apparatus that is slowly filled with redistilled solvent from a boiling flask/condenser system; and periodically, the extracting solvent (containing extractables) is siphoned back into the boiling flask and the process begins again (for as many times as required to attain equilibrium).
- *Sealed vessel*—in which the test article and extracting solvent are sealed inside a container capable of withstanding elevated temperatures and pressures, placed into a laboratory autoclave and heated with steam for a period of time.
- *Instrument-based solvent extraction*—in which the test article is placed inside a sealed apparatus and extracted in an automated cycle; examples include pressurized fluid extraction, microwave-assisted extraction, and supercritical fluid extraction.
- *Sonication*—in which the test article and extracting solvent are placed into a glass container and partly immersed in water inside an ultrasonic bath.

Each of these extraction mechanisms/techniques has its own unique advantages and limitations. For example, reflux extraction is very efficient, but may be too harsh for certain applications and can lead to thermal decomposition of certain organic extractables; the extracting power of sonication can be difficult to control; and because of its relatively high boiling point, water performs poorly in reflux and Soxhlet but well in a sealed vessel.

If the goal of the extractables assessment is identification and quantitation of the chemical additive content of a component or material, it is typical to use extraction techniques and processes that soften, swell, or dissolve (or in the case of inorganic extractables, *digest*) the component or material, thereby releasing quantitative amounts of chemical additives for analysis.

Extractions That are Not Solvent Mediated

Not all drug product or material-contact situations are solution mediated and not all issues related to leaching of material-derived entities involve a solution phase. For example, doses of inhalation powder contained in a capsule or blister pack for use in a dry powder inhaler may have volatiles leached from the capsule or blister material, or by specialty surface additives such as mold-release agents; a solid oral dosage form could contain volatile leachables derived from the adhesive of a

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paper label affixed to the plastic bottle that contains the dosage form; and inhalation solutions packaged in low-density polyethylene containers could contain volatile migrants from tertiary packaging or auxiliary components such as wooden shipping pallets. In the latter two cases, chemical entities can migrate through the plastic containers and volatilize into the airspace, subsequently accumulating as migrants in the dosage forms.

Extraction techniques specifically designed for application to volatile organic compounds are usually directly coupled to analytical instruments. These extraction techniques include headspace analysis (as headspace gas chromatography; HD/GC), direct thermal desorption (usually coupled to gas chromatography; TD/GC), and thermogravimetric analysis (TGA/GC).

CHARACTERIZING THE EXTRACT

Objectives and Challenges

Once an extract has been generated, the next objective is to perform a thorough chemical characterization of the extract. Setting a threshold (as described above), which is a specified level of an individual extracted chemical entity which requires characterization, can be based on safety considerations such as the SCT; functional considerations including nominal levels of known chemical additives in the formulation of an extracted component or material; or technological considerations such as the known or determined sensitivity of an analytical technology, instrument, or method. The extract characterization phase of the extraction study must enable the realization of the overall goals of the extractables assessment.

The ultimate objective of thorough extract characterization as defined above cannot be realized in all cases, even when state-of-the-art analytical chemistry is practiced with best available skill and diligence. It is a reality that there is no analytical technique or combination of analytical techniques that is capable of the discovery, identification, and quantitation of any and all organic and inorganic extractable chemical entities known to science. In some cases, authentic reference compounds for organic extractables may not be available for confirmation of identifications, or for quantitative instrument calibration. Thus, the practical objective of extract characterization must therefore be an exercise of due diligence in the discovery, identification, and quantitation to a reasonable degree of scientific certainty of all individual extractable chemical entities present in an extract above a specified level or threshold.

Processes Involved in Extract Characterization

1. SCOUTING

The most useful analytical techniques in a scouting exercise are not compound specific, as they do not provide chemical information specific to the molecular structure of any particular extractable or chemical class of extractables. These analytical techniques provide information regarding bulk chemical properties of organic and/or inorganic chemical entities present in an extract, which can be used to guide extractables discovery, identification, and quantitation. Scouting analysis is not capable in and of itself of realizing the practical objective of extract characterization, regardless of which scouting technique or combination of techniques is applied.

Analytical techniques which can be employed for scouting are listed in *Table 3*, along with the particular bulk chemical property (and potential utility of this property) available from each technique. Some examples of the utility of scouting include the following:

- Significant levels of nonvolatile residue determined by gravimetric analysis could suggest the presence of significant levels of inorganic chemical entities in the extract. This suggestion would be reinforced if significant mass remained after ashing the extracted nonvolatile residue (residue on ignition).
- Significant UV absorbance of an extract suggests that organic chemical entities are present which contain UV chromophores within their molecular structure, such as phenolic antioxidants.
- Characteristic features in an infrared spectrum of this extract could provide more detailed insights into the chemical classes of organic extractables present. These insights could be used to develop and apply analytical methods for discovery and identification that would detect the chemical classes of extractables suggested by the scouting process.
- For aqueous extracts, total organic carbon provides a measure of the total amount of organic extractables present.

The scouting process and scouting analyses are optional for extract characterization. The utility of scouting is in the guidance it potentially provides for discovery, identification, and quantitation.

Table 3. Survey of Analytical Methods for Extract Analysis

Analytical Technique	Analytical Method	Application				Information/Utility
		Scouting	Discovery	Identification	Quantitation	
Spectroscopy	UV	X		X		Bulk property of UV absorbing organic extractables; semi-quantitative with limited identification ability
	FTIR[a]	X		X		Bulk property of IR absorbing organic extractables, moderate identification ability
Wet Chemical	NVR[b], ROI[c]	X				Bulk property reflecting total amount of nonvolatile organic and/or inorganic extractables
	pH	X				Bulk property of acidic or basic extractables
	TOC[d]	X				Quantitative measure of organic extractables

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Table 3. Survey of Analytical Methods for Extract Analysis *(continued)*

Analytical Technique	Analytical Method	Application				Information/Utility
		Scouting	Discovery	Identification	Quantitation	
Gas Chromatography	FID[e]		X	X	X	Discovery and quantitative assessment of individual organic extractables; note that qualitative identification is possible
	MS		X	X	X	Discovery, identification, and quantitation of individual organic extractables; note that identification can be either qualitative or structural
	FTIR[a]		X	X		Discovery and identification of individual organic extractables; note that FTIR has limitations relative to structural analysis (however identification via qualitative analysis is possible)
Liquid Chromatography	UV, CAD[f], and ELSD[g]		X		X	Discovery and quantitative assessment of individual organic extractables; note that identification via qualitative analysis is possible and that Diode Array UV detectors can assist with structural analysis
	MS		X	X	X	Discovery, identification, and quantitation of individual organic extractables; note that identification can be by either qualitative or structural and that ionization sources with different selectivities are available
	FTIR[a]		X	X		Discovery and identification of individual organic extractables; note that FTIR has limitations relative to structural analysis (however identification via qualitative analysis is possible)
	NMR[h]		X	X		Identification of individual organic extractables; note that identification can be by either qualitative or structural
Ion Chromatography	Conductivity		X		X	Discovery and quantitation typically of individual ionic species
	MS		X	X	X	Discovery, identification, and quantitation of individual ionic extractables; note that identification can be by either qualitative or structural and that ionization sources with different selectivities are available
Spectrometry	MS			X		Identification of individual organic extractables
	NMR[h]			X		Identification of individual organic extractables
	IMS[i]		X	X	X	Discovery and quantitative assessment of individual organic extractables; note that various ionization sources are available and that qualitative identification is possible
Atomic Spectroscopy	AAS[j]		X	X	X	Discovery, identification, and quantitation of individual extracted elements (trace elements, metals); note that AAS can be applied to only one element at a time. Identification of the chemical form or speciation of the extracted element may require additional testing
	ICP-AES[k]		X	X	X	
	ICP/MS[l]		X	X	X	

[a] FTIR = Fourier Transform Infrared spectroscopy.
[b] NVR = Nonvolatile Residue.
[c] ROI = Residue on Ignition.
[d] TOC = Total Organic Carbon.
[e] FID = Flame Ionization Detection. Additional GC detectors, such as Thermal Energy Analysis Detector (TEA), may provide greater sensitivity for specific compound classes.
[f] CAD = Charged Aerosol Detector.
[g] ELSD = Evaporative Light Scattering Detector.
[h] NMR = Nuclear Magnetic Resonance spectroscopy.
[i] IMS = Ion Mobility Spectrometry.
[j] AAS = Atomic Absorption Spectroscopy.
[k] ICP-AES = Inductively Coupled Plasma Atomic Emission Spectroscopy.
[l] ICP/MS = Inductively Coupled Plasma Mass Spectrometry.

2. DISCOVERY

The process of discovery involves testing an extract and thereby producing one or more analytical results that are attributable to individual extractables. The process of discovery is accomplished by detecting instrumental responses from the individual organic and inorganic extractables that are proportional to the levels of these individual extractables within the extract. It is in the discovery process that analytical techniques typically associated with trace organic and inorganic analysis are first required for extract characterization. Trace organic analysis typically involves the use of chromatographic techniques, particularly gas chromatography (GC) and high-performance liquid chromatography (HPLC). GC has enormous separating capability for volatile and semi-volatile organic compounds while HPLC is most applicable to semi-volatile and relatively nonvolatile organic

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compounds, making the two separation techniques complementary and orthogonal for application to the significant chemical diversity of extractables. A discussion of the principles of both gas and liquid chromatography is available in *Chromatography* ⟨621⟩.

The chemical diversity of extractables with respect to polarity and volatility can require alternative sample introduction techniques or sample modification, particularly for GC. Relatively volatile extractables like methanol are most amenable to headspace sampling of aqueous-based extracts into a GC. Organic acids and bases can often be analyzed more effectively by GC after chemical derivatization, such as methylation or silylation for organic acids. Both GC and HPLC can employ detection systems with different specificities (*Table 3*) which take advantage of unique structural properties of various chemical classes of extractables.

The analytical techniques useful for organic extractables discovery can also be applied to identification as well as quantitation. Analytical techniques such as gas chromatography/mass spectrometry (GC/MS), that are most often applied to identification, can also be used for both discovery and quantitation (*Table 3*). Inorganic extractables such as trace elements and metals are typically discovered, identified, and quantitated by the same suite of analytical techniques, such as atomic emission spectroscopy. Analytical techniques designed to study inorganic speciation, particularly in aqueous extracts, are considered beyond the scope of this chapter.

It is important to state that the overall goals of an extraction study always require the identities and quantitative amounts of individual organic and inorganic extractables, and so the mere discovery of extractables does not achieve the ultimate objectives of an extraction study.

3. IDENTIFICATION

Identification of an extractable can be accomplished either by structural analysis or qualitative analysis. *Structural analysis* is the process by which the molecular structure of an unknown analyte is elucidated from compound-specific data, and therefore requires compound-specific detection of the unknown analyte. A compound-specific detector is one that provides information specific to the molecular structure of the individual unknown analyte (not just its chemical class). *Qualitative analysis* is the process by which an unknown analyte is matched with an authentic reference compound via one or more analytical techniques. The analytical techniques used for qualitative analysis can, but do not need to be, compound specific.

The analytical techniques most applicable to structural analysis, and to trace organic analysis problems in general, involve the combination of chromatography with mass spectrometry. These are the so-called "hyphenated" techniques of GC/MS and high-performance liquid chromatography/mass spectrometry (LC/MS). A discussion of the principles of mass spectrometry (including both GC/MS and LC/MS) is available in *Mass Spectrometry* ⟨736⟩.

Both GC/MS and LC/MS are capable of generating extractables profiles in the form of chromatograms. However, since LC/MS includes a relatively high chemical background of HPLC mobile phase ions, it is typical to include a non-destructive UV detector in series with the mass spectrometer to assist in locating peaks of individual extractables. The compound-specific data available from mass spectrometry include:

- The monoisotopic molecular weight of the extractable based on confirmation of the molecular ion from one or more ionization processes
- The molecular formula of the extractable based on accurate mass measurements, and/or accurate isotope ratio measurements, of the molecular ion
- The fragmentation behavior of the extractable based on in-source fragmentation or tandem mass spectrometry

GC/MS interfaced with electron ionization produces mass spectra which can be searched through computerized databases, or libraries, of mass spectra from known compounds. Note that searchable mass spectra are generally unavailable for LC/MS ionization processes because of the variable nature of such spectra over time and between various instruments and laboratories. Both GC/MS and LC/MS also include the retention time (or retention index) of the unknown extractable which can be compared with that of authentic reference compounds.

Given the number and chemical diversity of organic extractables, it is unreasonable to expect that authentic reference compounds will be available (or can be made available) to confirm every identification. It is therefore necessary that levels of identification confidence be established and appropriately utilized. Data typically available from GC/MS and LC/MS analyses (see items a through e below) are used to designate individual extractables identifications in the categories of *Confirmed*, *Confident*, or *Tentative* (*2*):

- Mass spectrometric fragmentation behavior/expert mass spectrum interpretation
- Confirmation of molecular weight
- Confirmation of elemental composition
- Mass spectrum matches automated library or literature spectrum
- Mass spectrum and chromatographic retention index match authentic reference compound
- Supporting spectral information from an orthogonal method (e.g., NMR)
    - A *Tentative* identification means that data have been obtained that are consistent with a class of molecule only. This is typically the case when only information such as a or d is available.
    - A *Confident* identification means that the tentative identification has been bolstered by additional and sufficient confirmatory information to preclude all but the most closely related structures. This would be the case, for example, if the tentative information (a and/or d) were augmented by b, c, or f. The more confirmatory information obtained, the greater the level of confidence.
    - A *Confirmed* identification means that the preponderance of evidence confirms that the entity in question can only be the identification that is provided. Although it is possible that a highly confident identification may meet the standard implied by the preponderance of evidence (for example, having a, b, c, e, and f), the only means of providing a confirmed identification is via mass spectral and retention time match with an authentic reference compound (item e).

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Although these identification categories are based on mass spectrometry, it is possible to use data from other analytical techniques to assist in extractables identification. Such techniques include GC/FTIR (Fourier Transform Infrared Spectroscopy) and LC/NMR (Nuclear Magnetic Resonance Spectroscopy). These and potentially other analytical techniques are capable of producing compound-specific data which are complementary to mass spectrometry.

The level of identification required for any individual extractable depends on the intended use of that identification. It is up to the organization responsible for the extraction study to determine this after appropriate consideration of applicable regulatory guidances.

Since the list of potential inorganic extractables, such as trace elements and metals, is finite compared with the population of organic extractables, identification and quantitative analysis for inorganic extractables are achieved simultaneously. While elemental analysis is relatively straightforward, it is not without its challenges. The issue of false positive responses and spectral or mass interference must be addressed in order for identifications based on atomic spectroscopy to be rigorous and accurate. It is also noted that elemental analysis provides element-specific identifications and quantitations, and not the chemical speciation of the extractable. Thus, interpretation of the impact of the elemental results may require further studies, such as detailed chemical speciation of elements deemed of significance. For example, while finding sulfur in an extract by atomic spectroscopy is an important outcome, the safety impact of this finding cannot be ascertained until the speciation of the sulfur is established. This is the case as the safety impact of sulfur as elemental sulfur may be different than that of sulfur as sulfate.

4. QUANTITATION

Quantitation is typically based on the instrumental response of an individual extractable relative to an authentic reference compound, and therefore requires that individual extractables be separated (either directly with chromatography or indirectly with selective detection) and produce detector responses that are directly proportional to the level (or concentration) of the extractable in a given extract. Calibration of an analytical system is accomplished by analysis of authentic reference compounds (external standards). One or more internal standards can also be included in both the extract and reference calibration solutions to increase accuracy and precision. The levels of extractables for which authentic reference compounds are not available can be estimated using their responses (or response factors) relative to internal standards, or other surrogate reference compounds of similar molecular structure. While such an analytical process can provide reliably accurate concentration estimates, diligence must be exercised in terms of establishing and justifying the choice and use of internal standards. Criteria for the selection of appropriate internal standards have been described (*2*).

Preparation of Extracts for Analysis

Extracts can often be analyzed directly without significant preparation or concentration. Many organic solvent extracts (e.g., dichloromethane, ethyl acetate, hexane) can be directly injected into a gas chromatograph, while others (e.g., methanol, ethanol, isopropanol) are either too reactive in the heated GC injection port or too high boiling. Organic solvent extracts with inappropriate physical/chemical properties for direct analysis by GC can be switched to more appropriate solvents. Certain extractables, such as fatty acids (e.g., palmitic acid, stearic acid) perform better in gas chromatographic analysis when they are derivatized to either methyl esters or trimethylsilyl esters.

It is usually considered inappropriate to directly analyze aqueous extracts by gas chromatography, due to the reactivity and high boiling point of water. In addition, pH-buffered aqueous extracts contain nonvolatile salts which are not suitable for GC injection. Aqueous extracts are typically back-extracted with an organic solvent to remove organic extractables from the water, with the resulting organic extract being injected into the GC. Unlike GC, liquid chromatography (HPLC, LC/MS) is perfectly suited to the direct analysis of aqueous extracts, since most HPLC methods include water and water-miscible mobile phases. Water-immiscible organic solvents (e.g., hexane) cannot be injected onto these reversed-phase HPLC systems, so these must be dried and the resulting extractable residue taken up in a solvent suitable for HPLC (e.g., acetonitrile, methanol, or mixtures of these with water).

Organic or aqueous extracts with insufficient levels of extractables for analysis can be concentrated by various techniques. Many organic solvents can be dried down under inert gas, a rotary evaporator, or a Kuderna-Danish concentrator. Aqueous extracts can be lyophilized, concentrated under vacuum, or back extracted into an organic solvent which is then further concentrated.

The final concentration at which an extract is analyzed depends on the goal(s) of the extractables assessment and the inherent sensitivities of the analytical techniques applied. A good "rule of thumb" is that in order to accomplish a complete structural analysis of an unknown extractable, a GC/MS requires approximately 5 ng injected into the instrument. This suggests a concentration in the injected extract of 5 ng/µL or 5 µg/mL. In a 200-mL dichloromethane extract, this converts to a total of 1 mg of this particular extractable recovered from the extracted test article. If this analyte concentration is insufficient to meet the goal of the extractables assessment, then the following parameters can be optimized:

- Extraction stoichiometry (i.e., extract more material or use more extracting solvent)
- Extraction conditions (i.e., use higher temperatures, longer times, solvents with greater extraction power, more aggressive extraction technique, etc.)
- Extract processing (i.e., concentration of the extract)

SUMMARY

Assessing the Completeness of an Extractables Assessment

The completeness of an extractables assessment can only be judged against the overall goals of the assessment. For example, an extractables assessment accomplished solely for materials characterization might include one extracting solvent, one

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extraction technique, and one set of extraction conditions; along with a materials-based threshold (e.g., 10 ppm w/w). Such an extractables assessment might be considered complete if all extractables above the defined threshold were identified to the confident level (defined above) and quantitated. For an extractables assessment designed to establish a rigorous leachables–extractables correlation for a high risk drug product, where a challenging safety threshold might apply (e.g., 0.15 µg/day), good scientific practice and due diligence requires the following:

- Generation of extracts should be accomplished with
  - Multiple solvents or extracting media with varying extracting power based on the known extracting power of the drug product vehicle;
  - Multiple and complementary extraction techniques, including those with the capability for volatiles analysis;
  - Extraction conditions that allow equilibrium to be achieved.
- Characterization of extracts should use
  - Multiple and complementary analytical techniques;
  - Careful sample preparation, keeping the analytical technique(s) in mind;
  - A systematic process for identification and quantitation of extractables.

In this case, the extractables assessment might be considered complete if all extractables above the defined threshold were identified to at least the confident level, quantitated, and correlated both qualitatively and quantitatively with drug product leachables data (if available) and the known ingredients in the packaging system, packaging component(s), or material(s) of construction.

It should be noted that limited extractables assessments with relatively narrow goals can be accomplished to required completeness with a relatively focused effort. For example, extraction studies designed to quantitate the levels of specific chemical additives in specific packaging components/materials can be done with specified extraction parameters and analytical methods (see *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩). The reader is also referred to various sources which describe extractables assessments and extraction studies for pharmaceutical applications (*2*, *5*), as well as other general sources which refer to extractables assessments for medical devices and food contact (*8*).

Reference is also made to compendial chapters in this Pharmacopeia which include extraction studies with specific goals and purposes:

1. *Biological Reactivity Tests, In Vitro* ⟨87⟩
2. *Biological Reactivity Tests, In Vivo* ⟨88⟩
3. *Elastomeric Closures for Injection* ⟨381⟩
4. *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩
5. *Plastic Materials of Construction* ⟨661.1⟩
6. *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩

Example Extractables Profiles—Materials Characterization

As stated previously, extraction studies are usually designed to produce extractables profiles, which are qualitative and/or quantitative analytical representations of the extractables content of a particular extracting medium. To illustrate the concept of an extractables profile, the following is an example of an extractables study performed for the purpose of material characterization. Extractables profiles are commonly produced by analysis of laboratory extracts by instrumental chromatographic techniques. *Figure 2* and *Figure 3* show example extractables profiles (GC/MS and HPLC/UV, respectively) from a hexane Soxhlet extract of a cyclic olefin copolymer (COC) material of construction. COC materials are used in the fabrication of pre-filled syringes, vials for small-volume parenterals, and bags for large-volume parenterals. The extracts were generated by subjecting approximately 5 g of suitably sized material to 16 hours of Soxhlet extraction with 125 mL of hexane. The resulting extract was spiked with internal standards (details not relevant to this discussion) and was analyzed directly by GC/MS (see *Table 4*). For HPLC/UV analysis, an aliquot of the hexane extract was reduced in volume and diluted with methanol prior to analysis (see *Table 5*). Since the purpose of this extractables assessment was materials characterization, extraction study parameters were adjusted relative to an extractables identification threshold of 10 ppm (µg/g). It is clear from these chromatograms that the techniques used are both complementary and orthogonal, illustrating the concept that multiple analytical methods are required to typically elucidate the complete extractable profile.

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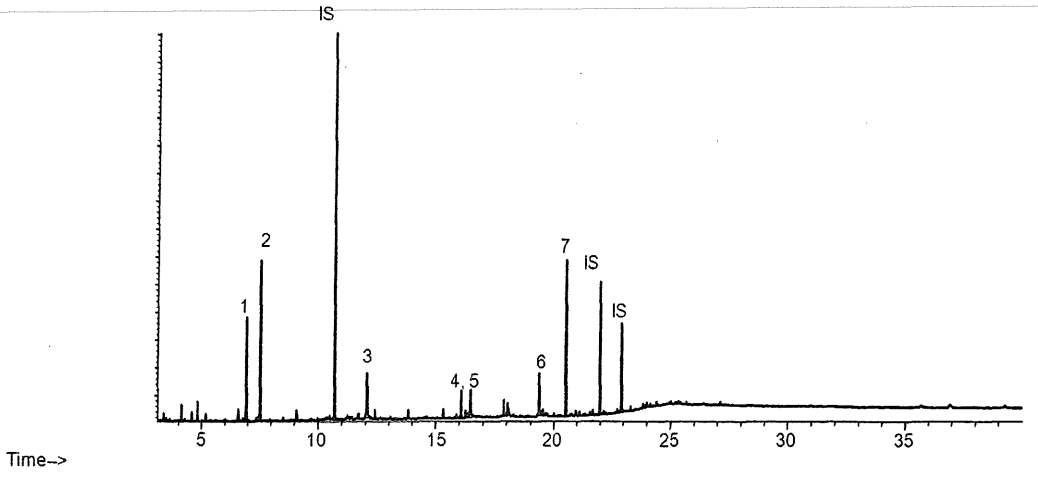

Figure 2. GC/MS chromatogram (extractables profile) for a hexane Soxhlet extract of a cyclic olefin copolymer. Internal standards (IS) producing peaks in this chromatogram include: 2-Fluorobiphenyl at 10.7 min, Irganox 415 at 22.0 min, and Bisphenol M at 23.0 min. Numbered peaks represent identified extractables above the materials-based threshold.

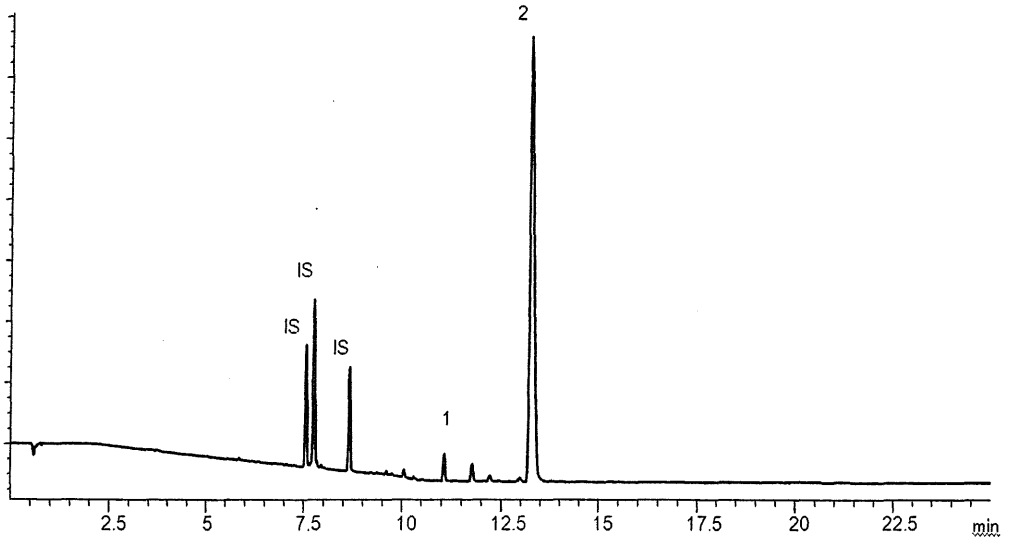

Figure 3. HPLC/UV Chromatograms (λ = 220 nm; extractables profile) for a hexane Soxhlet extract of a cyclic olefin copolymer. Internal standards (IS) producing peaks in this chromatogram include: Bisphenol M at 7.6 min, 2-Fluorobiphenyl at 7.8 min, and Irganox 415 at 8.7 min. The major peak in this extractables profile above the materials-based threshold is the known additive (antioxidant) Irganox 1010 (Peak 2).

Table 4. Operating Parameters, GC/MS Analysis of the Hexane COC Soxhlet Extract

Operating Parameter	Operating Value
Column	J&W DB-5HT, 30-m × 0.25-mm, 0.25 µm film thickness
Oven Program	Start at 50°, hold for 1 min; ramp at 12°/min to 315°, hold for 16 min
Carrier Gas	He at 1.2 mL/min
Injection	Split (1:5); 1 µL

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Table 4. Operating Parameters, GC/MS Analysis of the Hexane COC Soxhlet Extract *(continued)*

Operating Parameter	Operating Value
Injector Temperature	300°
FID Detector Temperature	N/A
MS Transfer Line Temperature	180°
MS Detection Details	70 eV (+) EI (electron ionization), mass range of 33–650 amu (3.0-min solvent delay)

Table 5. Operating Parameters, HPLC/UV Analysis of the Hexane COC Soxhlet Extract

Operating Parameter	Operating Value	
Column	Agilent Zorbax Eclipse Plus C_{18}, 100- × 3.0-mm, 3.5-µm particles	
Column Temperature	40°–50°	
Mobile Phase Components	A = 10 mM ammonium acetate, B = acetonitrile	
Mobile Phase Gradient	Time	%B
	0.0	5.0
	8.4	100.0
	35.0	100.0
	36.0	5.0
	39.0	5.0
Mobile Phase Flow Rate	0.8 mL/min	
Sample Size	10–50 µL	
Detection, UV	205–300 nm; spectra recorded at λ = 210, 220, 230, 250, and 270 nm	

Change to read:

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⟨1664⟩ ASSESSMENT OF DRUG PRODUCT LEACHABLES ASSOCIATED WITH PHARMACEUTICAL PACKAGING/DELIVERY SYSTEMS

PURPOSE

This general chapter presents a framework for the design, justification, and implementation of assessments for drug product leachables derived from pharmaceutical packaging and delivery systems. A scientifically sound leachables assessment is important to manufacturers and their various suppliers primarily as a means of establishing the suitability for use of pharmaceutical packaging/delivery systems, as leachables can potentially affect drug product efficacy, safety, and quality. Additionally, such a leachables assessment could provide an understanding of the sources of leachables and how to evaluate and manage leachables during the drug development and manufacturing processes. The chapter establishes critical dimensions of a leachables assessment and discusses practical and technical aspects of each dimension. The chapter does not establish specific analytical methods or leachables specifications and acceptance criteria for any particular dosage form or packaging

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system or drug product combination; nor does it delineate every situation in which a leachables assessment is required. It is not possible for a general discussion of drug product leachables to anticipate and cover all situations which can occur in the pharmaceutical industry where a leachables assessment might be required. Designing an individual leachables assessment is a process that strikes a balance between sound science, prudent resource allocation, and effective risk management with an emphasis on patient safety and product quality. Achieving this balance is the responsibility and obligation of the drug product manufacturer, and assumes due consideration of applicable legal and regulatory requirements. The principles and best demonstrated practices outlined in this general chapter represent a consensus interpretation of sound science and can therefore be extrapolated and applied to any situation in which a leachables assessment is required for pharmaceutical application.

In many cases, drug product leachables assessments are based on or facilitated by knowledge from extractables assessments accomplished on drug product packaging systems, packaging components, and packaging materials of construction (see *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩).

KEY TERMS

This general chapter uses the following key terms (*1,2*; also see *Packaging and Storage Requirements* ⟨659⟩). Note that the terms *Packaging System*, *Packaging Component*, *Primary Packaging Component*, *Secondary Packaging Component*, and *Materials of Construction* are also defined in ⟨659⟩, and the definitions below are intended for clarification purposes within the context of this chapter and are not intended to supersede those provided in ⟨659⟩.

Packaging Systems are the sum of packaging components that together contain and protect the dosage form. Packaging systems are also referred to as *Container Closure Systems* and may include primary, secondary, and tertiary packaging.

A *Container* is a receptacle that holds an intermediate compound, active pharmaceutical ingredient, excipient, or dosage form and is in direct contact with the product.

A *Closure* is a material that seals an otherwise open space on a container and provides protection for the contents. It also provides access to the contents of the container.

A *Packaging Component* is any single part of the package or container–closure system including the container (e.g., ampuls, prefilled syringes, vials, bottles), closures (e.g., screw caps, stoppers), ferrules and overseals, closure liners, inner seals, administration ports, overwraps, adminstration accessories, labels, cardboard boxes, and shrink wrap.

A *Primary Packaging Component* is in direct contact or may come into direct contact with the product (e.g., IV bag).

A *Secondary Packaging Component* is in direct contact with a primary packaging component and may provide additional protection of the product (e.g., overpouch or dustcover for an IV bag).

A *Tertiary Packaging Component* is in direct contact with a secondary packaging component and may provide additional protection of the product during transportation and/or storage (e.g., shipping carton for an overpouched IV bag).

An *Ancillary Component* is a component or entity that may come into contact with a tertiary packaging component during the distribution, storage, and transportation of the packaged product (e.g., pallets, skids, shrink wrap, active containers).

Packaging Materials of Construction are substances used to manufacture packaging components. These are also referred to as *Raw Materials*.

A *Delivery System* is the sum of components and materials that are used to transport a drug product from its packaging to the point of administration into the patient. For example, an administration set is a delivery system that is used to transfer liquid drug products from their plastic packaging system to the site of administration to the patient.

Extractables are organic and inorganic chemical entities that can be released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction and into an extraction solvent under laboratory conditions. Depending on the specific purpose of the extraction study (discussed below), these laboratory conditions (e.g., solvent, temperature, stoichiometry, etc.) may accelerate or exaggerate the normal conditions of storage and use for a packaged dosage form. Extractables themselves, or substances derived from extractables, have the potential to leach into a drug product under normal conditions of storage and use and become leachables. Thus extractables are potential leachables.

Leachables are foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. Because leachables are derived from the packaging or delivery system, they are not related to either the drug product itself or its vehicle and ingredients. Leachables are present in a packaged drug product because of the direct action of the drug product on the source of the leachable. Thus leachables are typically derived from primary and secondary packaging, because the primary and secondary packaging can serve as a barrier between the packaged drug product and other potential sources of foreign chemical entities (e.g., tertiary packaging and ancillary components). In certain circumstances, packaging may directly contact the patient under typical clinical conditions of use (e.g., the mouthpiece of a metered dose inhaler). As a result of this contact, patients may be exposed to leachables from the packaging without the action of the drug product. Leachables are typically a subset of extractables or are derived from extractables. Note that chemical entities can also migrate from packaging/delivery systems to patients via direct contact.

Migrants are also foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. However, migrants are differentiated from leachables by the circumstance that migrants accumulate in the packaged drug product after the migrant has crossed a physical barrier, such as that provided by primary and secondary packaging. Because migrants cross a physical barrier, they are not present in the packaged drug product due to direct action of the drug product on the source of the migrant because the barrier prevents such direct action. Thus migrants are derived from secondary and tertiary packaging and ancillary components.

Regardless of whether a substance is a leachable or migrant, it is still a foreign substance in the packaged drug product, and thus its impact must be assessed in the same manner. However, as the means by which a leachable and a migrant become

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entrained in a packaged drug product may be different, extractables studies meant to address leachables may be designed and implemented differently than extractables studies meant to address migrants.

Leachables Studies are laboratory investigations into the qualitative and quantitative nature of a particular leachables profile(s) over the proposed shelf-life of a particular drug product.

Characterization is the discovery, identification, and quantitation of each individual organic and inorganic leachable present in a drug product formulation above a predetermined level or threshold. Such thresholds should be based mainly on patient safety considerations, with consideration also given to the capabilities of analytical technology, and other related issues.

Identification is the process of assigning a molecular structure to an organic leachable, or assigning constituent elements in the case of an inorganic leachable.

Quantitation is the process of measuring the level, or concentration, of an individual organic or inorganic leachable contained in a drug product formulation.

Leachables Profiles are qualitative and/or quantitative analytical representations of the leachables content of a particular drug product formulation.

Leachables–Extractables Correlations are established when observed drug product leachables are linked both *qualitatively* and *quantitatively* to extractables from associated packaging/delivery systems, packaging components, or materials of construction.

Threshold of Toxicological Concern (TTC) is a level of exposure for all chemicals, whether or not there is specific toxicity data, below which there would be no appreciable risk to human health (*6*). The TTC approach is a form of risk characterization in which uncertainties arising from the use of data on other compounds are balanced against the low level of exposure.

Safety Concern Threshold (SCT) is the threshold below which a leachable would have a dose so low as to present negligible safety concerns from carcinogenic and noncarcinogenic toxic effects.

Qualification Threshold (QT) is the threshold below which a given noncarcinogenic leachable is not considered for safety qualification (toxicological assessments) unless the leachable presents structure-activity-relationship (SAR) concerns.

Analytical Evaluation Threshold (AET) is the threshold at or above which a leachable should be characterized and reported for toxicological assessment. The AET can be mathematically derived from the SCT (or other threshold concepts) based on factors that include the dosing parameters of the drug product.

As noted, additional terminology and associated definitions are available (*1,2*; see also ⟨*659*⟩).

BACKGROUND

Management of leachables is important to pharmaceutical and biotechnology/biologic product manufacturers and regulatory authorities because certain leachables above specific concentrations can present safety concerns for patients and/or compatibility issues for drug product formulations. During the 1980s, the U.S. Food and Drug Administration (FDA) began to formally and comprehensively address leachables in drug products after findings of patient sensitivity induced by leachables and other potential safety concerns related to leachables (*2–4*). Since then, management of both extractables and leachables for packaging systems and final drug products has become an important part of pharmaceutical development and regulatory submissions for many dosage form types, particularly for those deemed of relatively high risk for dosage form interaction with the packaging system, along with a relatively high safety risk relative to the route of administration (see *Table 1*). Note that *Table 1* is a version of the original concept that appears in the FDA guidance *Container Closure Systems for Packaging Human Drugs and Biologics* (*1*), in which certain dosage forms in the above guidance have been downgraded to having lower potential for interaction with packaging components. Remaining relatively high-risk dosage forms include: inhalation aerosols and solutions, injectables and injectable suspensions, ophthalmics, and transdermal ointments and patches. It is important to note, however, that even low-risk dosage forms present some risk and that appropriately rigorous leachables assessments can be important to particular drug products in lower risk dosage form categories (e.g., topicals and oral dosage forms, etc.).

Table 1. Modified FDA/CDER/CBER Risk-Based Approach to Consideration of Leachables[a] (*1*)

Examples of Packaging Concerns for Common Classes of Drug Products			
Degree of Concern Associated with the Route of Administration	Likelihood of Packaging Component-Dosage Form Interaction		
	High	Medium	Low
Highest	Inhalation Aerosols and Sprays	Injections and Injectable Suspensions; Inhalation Solutions	Sterile Powders and Powders for Injection; Inhalation Powders
High	Transdermal Ointments and Patches	Ophthalmic Solutions and Suspensions; Nasal Aerosols and Sprays	—
Low	Topical Solutions and Suspensions; Topical and Lingual Aerosols; Oral Solutions and Suspensions	—	Oral Tablets and Oral (Hard and Soft Gelatin) Capsules; Topical Powders; Oral Powders

[a] While this table provides a convenient overview of the general level of regulatory concern with various dosage forms regarding leachables, it should not be inferred that "low-risk" dosage forms (e.g., oral tablets) by that definition carry no risk for leachables issues.

This chapter will describe scientific principles and best practices for the assessment of drug product leachables, and will cover various important concepts, including: 1) the requirement for leachables studies; 2) fundamental concepts for leachables studies; 3) the basis of thresholds for leachables and general guidance about application of these thresholds; 4) design and implementation of leachables studies; 5) leachables method development and validation; 6) correlation of results from extractables assessments and routine extractables testing with leachables studies; and 7) establishment of leachables specifications including acceptance criteria.

These scientific principles and best practices apply to all organizations and individuals involved in the manufacture, marketing, and qualification of drug products and in their stability studies, including but not limited to:

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- Manufacturers of drug products for human and veterinary use where manufacturing may involve operations at the applicant holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Manufacturers of combination drug products
- Packaging operations by the manufacturer or a designated contractor for the applicant holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer.

Although it is ultimately the drug product applicant's responsibility to ensure that appropriate leachables assessments are completed, manufacturers and fabricators of pharmaceutical packaging/delivery systems, packaging components, and materials of construction should also apply these scientific principles and best practices as appropriate, and applicants are encouraged to work with component manufacturers and fabricators to this end.

CONCEPTS

General Concepts for Leachables Assessment

During the course of manufacturing, packaging, storage, distribution, and administration, dosage forms and/or their formulation constituents contact components and materials of construction of manufacturing and packaging equipment, and primary and secondary packaging components and systems. Such contact may result in interactions between the dosage form and these components and materials. One such interaction is the migration, or leaching, of substances from any of these components and materials into the dosage form. Leachables, which can include both organic and inorganic (i.e., elemental) chemical entities with wide chemical diversity, are of concern due to their potential safety risk to patients and potential compatibility risks for the drug product. In order to assess these risks and manage the potential issues posed by leachables, it is necessary to know their identities and the levels to which they will accumulate in the finished drug product over its shelf-life. These two pieces of information can be used to establish the magnitude of patient exposure (dose) and therefore the safety risk posed by an individual leachable, as well as the likelihood of any drug product compatibility issues.

Regulatory guidelines, requirements, and various best practice recommendations all state that the definitive assessment of the potential impact of contact between a packaging/delivery system and a final dosage form involves testing the final drug product for leachables. In its most essential form, this impact assessment involves performing a migration, or leachables, study whose purpose is to discover, identify, and quantitate leachables that have migrated from the contacted system, components, or materials and accumulated in the finished dosage form under the product's actual manufacturing, storage and clinical use conditions. A leachables study is a laboratory investigation into the qualitative and quantitative nature of a particular leachables profile(s) over the proposed shelf-life of a particular drug product. The purpose of a leachables study is to systematically and rationally identify and quantify (i.e., characterize) drug product leachables to the extent practicable, and within certain defined analytical threshold parameters. The results of leachables studies are used in the overall leachables assessment to understand the impact of leachables on patient safety and drug product quality and stability.

Leachables studies can be used within the context of an overall leachables assessment to:

- Facilitate the timely development of safe and effective dosage form packaging/delivery systems, manufacturing systems, and processes by assisting in the selection of components and materials of construction
- Facilitate the establishment of qualitative and quantitative leachables–extractables correlations in drug products, when coupled with an appropriate extractables assessment(s)
- Establish the worst-case drug product leachables profile in a manner that facilitates the development of drug product leachables specifications and acceptance criteria (should these be required), and the safety evaluation/qualification of leachables
- Identify trends in drug product leachables accumulation levels over the shelf-life of a particular drug product
- Facilitate change-control processes for drug product packaging/delivery systems (as appropriate), packaging components, materials of construction, formulation constituents, etc.
- Facilitate investigations into the origin(s) of identified leachables whose presence causes out-of-specification (OOS) results for a marketed drug product.

In these ways, leachables studies and assessments can support Quality by Design (QbD) principles for the development and manufacture of pharmaceutical packaging/delivery systems and drug products.

A complete leachables assessment includes understanding the safety impact of individual leachables, safety qualification of individual leachables, and developing an understanding of the impact of individual leachables on drug product stability (i.e., compatibility) and stability. Although safety qualification is presented in general terms, the details of attaining this goal are beyond the scope of this chapter, which is limited to general scientific principles and best practices for the conduct of leachables studies and the other stated uses of the results of leachables studies within an overall leachables assessment. The reader is directed to authoritative manuscripts on this topic (*2, 8*).

Note that certain packaging and combination product medical device components are (or can be) in direct contact with a patient's mouth, nasal mucosa, or other body tissue(s) during normal use of the drug product. Such packaging components include metered dose inhaler and dry powder inhaler mouthpieces, transdermal patches, etc. Patients are potentially exposed to chemical entities by direct contact from such components. Assessment of patient exposure in such direct contact scenarios is best accomplished with appropriate extractables assessments and extraction studies, and the reader is referred to ⟨1663⟩.

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Safety Thresholds

Although leachables represent a particular class of drug product impurity, current regulatory guidance for drug product impurities specifically considers leachables to be out of scope (*5*). Thresholds that have been specifically proposed for drug product leachables are based on either patient safety considerations or the current capabilities of analytical technology. Safety thresholds are particularly important in a leachables assessment because current analytical technology allows detection of trace organic and inorganic chemical entities at extremely low levels (i.e., ng/mL; ng/g). Identification and risk assessment (or qualification) of every individual chemical entity in a typical leachables profile at the limits of current analytical technology is neither necessary from a toxicological perspective nor feasible in a typical drug product. Safety thresholds allow for a science- and risk-based determination of acceptable levels of leachables and can be based on established toxicological information, as well as additional safety risk factors that consider, e.g., route of administration, daily exposure, and treatment duration. Because safety thresholds are derived from exposure data they are considered in terms of units of exposure, such as Total Daily Intake (TDI). Thus, any safety threshold must be converted into units of concentration (e.g., µg/mL) so that it can be applied as an analytical threshold in the laboratory. The analytical threshold is a guide as to which chemical entities in the leachables profile should be considered for chemical characterization (i.e., confirmed identification) and safety evaluation and qualification.

An example of a safety threshold concept that has been practically applied in pharmaceutical development is the Threshold of Toxicological Concern (TTC) approach (*6*). The TTC concept was adopted by the European Medicines Agency (EMA) to evaluate genotoxic impurities, using an excess cancer risk factor of 10^{-5} (1 in 100,000) (*7*). The EMA's proposed safety threshold for genotoxic impurities using the TTC approach is 1.5 µg/day TDI. Other examples of safety thresholds include the Product Quality Research Institute (PQRI) Safety Concern Threshold (SCT) and Qualification Threshold (QT), derived and proposed for individual organic leachables in Orally Inhaled Nasal Drug Products (OINDP) (*2, 8*). The SCT is 0.15 µg/day TDI, and the QT is proposed at 5 µg/day TDI for an individual organic leachable. The development of the TTC approach provided a foundation, precedent, and guide for derivation of the PQRI SCT, which incorporates a 10^{-6} (1 in 1,000,000) risk factor rather than the 10^{-5} value used for the EMA threshold. This lower threshold was considered appropriate for leachables in OINDP because of considerations regarding the direct delivery of some of these dosage forms to diseased organs of a sensitive patient population, and assuming lifetime exposure. In addition, leachables are typically industrial chemicals with no direct structural relationship to any active ingredient or other formulation constituent. Below the SCT, identification and safety evaluation of leachables generally would not be required. Below the QT, leachables without structure alerts for carcinogenicity or irritation would not require compound-specific safety risk assessment. Note that neither the SCT nor the QT is a control threshold or safety-driven limit. Rather, they are leachables evaluation thresholds. The SCT in particular is designed to establish a threshold for characterization of unknown drug product leachables. Individual levels of safety concern, different from the SCT value, could be determined for known leachables and potential leachables (i.e., extractables).

For OINDP there are certain "special case" compounds and compound classes, that due to particular safety concerns (e.g., carcinogenic) were deemed to require lower thresholds based on the capabilities of specific analytical technologies and methods. These special case compounds for OINDP include: polyaromatic hydrocarbons or polynuclear aromatics (PAHs or PNAs), *N*-nitrosamines, and the individual chemical entity 2-mercaptobenzothiozole (see *Orally Inhaled and Nasal Drug Products* ⟨1664.1⟩).

Information Sharing

To successfully manage leachables throughout the drug product lifecycle, it is critical to establish close and regular communication among those stakeholders throughout the development and drug product lifecycle responsible for the quality of the drug product: chemists, toxicologists, packaging engineers, manufacturing operations, procurement, etc. With respect to leachables, communication between the analytical chemist and toxicologist is critical. For example, if a leachable is found to be above an accepted limit, or a new leachable is found, a safety evaluation will need to be performed. The chemist will need to provide the toxicologist with information that will help to qualify the leachable, including the identity of the leachable, which may include compound class or more specific information, such as chemical formula and structure; and the amount and concentration of the leachable in the drug product.

Information sharing between packaging component manufacturers/suppliers and drug product developers/manufacturers is also important in order to guide packaging component and materials of construction selection, provide knowledge of potential extractables and leachables, and facilitate leachables–extractables correlations via knowledge of packaging component chemical compositions, etc. (also see ⟨1663⟩).

LEACHABLES STUDY DESIGN

Although leachables studies may be accomplished at any time during the drug product development/manufacturing lifecycle, leachables studies are especially relevant during late stage product development or during formal product stability assessment. Ideally, leachables assessment is conducted as follows:

- The assessment is performed on the actual drug product and not simulations thereof (however, see *Simulation Studies*).
- The assessment is performed with the actual packaging and delivery system in the form it will be commercialized, not with a prototype or on system components.
- The related extractables assessments are accomplished on the same lots of packaging components used to manufacture the drug product lots on which the leachables assessments are performed.
- The assessment is performed on a product that is manufactured under conditions that reflect the actual commercial processes of production of the drug product and the packaging/delivery system, filling of the drug product into the packaging/delivery system, post-filling treatment of the filled packaging (e.g., terminal sterilization), distribution, storage,

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and clinical use of the drug product. Although leachables studies may include accelerated storage conditions, they cannot be limited to accelerated conditions and must include real-time assessment.

Leachables studies can also be performed early in the drug product development process (e.g., preclinical stage) in order to facilitate the selection of packaging components and their materials of construction. Such leachables studies are particularly useful for certain "high-risk" dosage forms (see *Table 1*) where selecting appropriate packaging components and materials of construction is critical. A variety of packaging components and materials of construction can be evaluated at the same time and drug product leachables profiles determined and evaluated for each configuration. For primary packaging systems or combination drug/device products this can be accomplished by using either the drug product formulation or a placebo formulation in contact with the proposed packaging system. In the latter case, the placebo formulation can be considered as a simulating solvent to characterize extractables as probable leachables (see *Simulation Studies*). In either case, the leachables study conditions (i.e., time, temperature, etc.) should be based on conditions that are relevant for either the use-life or shelf-life of the drug product. Preclinical development stage leachables studies can be designed in a systematic way in order to support QbD processes and principles. It is important to also note that during early stage drug product development for high-risk dosage forms, leachables characterization is recommended for any drug product batches that are used as test articles in any definitive toxicology or clinical studies. For "low-risk" dosage forms (e.g., solid orals, topical powders) leachables studies conducted throughout development might be appropriate in order to assess, and thereby avoid, problems with packaging systems that might appear either in later stage development or marketed product.

During later stage development of high-risk dosage forms in support of product registration, when the final market form of the packaged drug product is available, leachables studies may be accomplished on definitive registration batches of drug product during the course of overall product stability studies. The results of these leachables stability studies can be used to establish leachables–extractables correlations, identify trends in leachables accumulation levels, evaluate individual leachables and qualify them on a safety basis, and develop leachables specifications with acceptance criteria (should these be required). For inhalation aerosols and other OINDP, leachables testing should be an integral part of the larger ICH registration stability program (*2*), and storage conditions and stability time points should be planned accordingly. For cases where a packaging/delivery component is in direct contact with the patient (e.g., a metered dose inhaler or dry powder inhaler actuator mouthpiece), chemical entities that a patient might be exposed to can be evaluated as extractables (i.e., potential leachables) using appropriate simulating fluids under time/temperature exposure conditions relevant to the intended use (see ⟨1663⟩).

Additionally leachables assessments may be appropriate on certain occasions post-market. For example, drug product leachables studies may also be appropriate in many cases where necessary or desired changes in a marketed drug product are made. Such leachables studies are normally required to support change-control processes for many high-risk dosage forms, particularly those with in-place leachables specifications and acceptance criteria, and could also be appropriate for other dosage form types, drug/device combination products, etc. Changes may include but are not limited to: composition of the drug formulation; manufacturing processes for the drug product; primary and secondary packaging components or their materials of construction; manufacturing or assembly processes for primary and secondary packaging components or their materials of construction; and delivery system(s) that are part of the drug product labeling. Any change that results in the patient being exposed to a different leachables profile than the one approved during registration will require leachables studies as part of any change-control process unless adequate scientific justification is provided to the contrary.

Although low-risk dosage forms (e.g., solid orals, topical powders) typically do not rigorously require leachables studies as part of the drug product registration process (*Table 1*), it is possible that leachables could appear in drug product impurity profiles either during registration stability studies or in marketed products. For example, it has been documented that chemical additives in label adhesives can migrate through plastic primary packaging and appear in impurity profiles of solid oral dosage forms packaged within these containers. Thus, it is appropriate to consider performing leachables studies on "low-risk" dosage forms in certain cases. If leachables assessment is not performed proactively, such an event could lead to an OOS result for a development or marketed product and require an "emergency" leachables study as part of an investigation process. The design of this type of leachables study depends on the particular situation; however, in general it would be necessary to identify and quantify the leachable(s), evaluate safety and possibly qualify the leachable(s), and correlate the leachable(s) with packaging component extractables. It is also possible that leachables could result from contact with manufacturing equipment and tertiary packaging systems (e.g., shipping materials).

The design of a particular leachables study depends on the purpose and goals of the overall leachables assessment. Although the leachables studies described above have different purposes and overall goals, they require similar types of information for their proper design. First, it is important to have information as to the identities and maximum possible accumulation levels of all potential leachables. The packaging component manufacturers may provide chemical composition details for the packaging/delivery system and various materials of construction, as well as details regarding the manufacturing processes for these components and materials. Such information may be in the form of material safety data sheets, technical data sheets, test reports, or confidential communications, and can be used to infer potential leachables. An extractables assessment (including an extraction study) can also be accomplished on packaging components and/or their materials of construction to directly assess potential leachables (see ⟨1663⟩). Regardless of how the chemical information is obtained, it is important to ensure that all possible sources of potential leachables from the finished packaging system are considered. These may include chemical entities from any of the primary and secondary packaging components and their materials of construction, coatings, cleaning, lubricating, cutting, sterilization, assembly, or other processes associated with the manufacture of the final packaging/delivery system as used in the drug product. The chemical information on the packaging and delivery system is used to create a list of potential leachables and their possible accumulation levels.

Potential leachables have a significant chemical diversity, and therefore a diversity of physical and chemical properties, including polarity, volatility, solubility, etc. Whereas relatively volatile compounds can more readily migrate into any type of formulation through indirect contact, nonvolatiles generally require direct contact. Two aspects of formulation contact should be considered: the nature of the formulation contact (i.e., direct or indirect) and the time of contact (transient or continuous). If the formulation is not in direct contact with the packaging component (e.g., inhalation powder in a capsule packaged in a blister) then it is less likely that any relatively nonvolatile compounds would migrate into the formulation from the packaging system; however, volatile compounds might. If the formulation is only briefly contacting the packaging component (e.g., an

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inhaler mouthpiece) it is less likely that any migration of chemicals from the component would occur on this transient timescale. However, if the formulation is in continuous contact with the packaging component (e.g., parenteral in a bag delivered through an administration set) then all types of compounds could potentially migrate into the formulation.

A rigorous leachables assessment considers leachables from sources other than primary packaging, such as necessary secondary packaging and, in certain situations, tertiary packaging. If the primary packaging consists of a semipermeable polymer (e.g., a low density polyethylene container), then potential leachables from labels, inks, adhesives, etc. that are used in the secondary packaging must be evaluated. Similarly, volatile compounds that are present in tertiary packaging (e.g., wooden pallets, cardboard boxes, plastic overwraps, etc.) could migrate into a formulation contained in such a plastic bottle. These migrants that are derived from tertiary packaging should be considered in the event that an unknown impurity is detected or suspected in the drug product.

Various characteristics of the drug product formulation must also be considered in designing any leachables study. For example, formulations are typically either solids or liquids, and it is well documented that physical state affects the leaching process. In the event that a formulation has a change in state during the course of production (e.g., lyophilization; liquid to solid) then the leachables study should be designed taking into consideration the time periods that the formulation is expected to be in each physical state. In the event that a formulation has a change in state during the course of use (e.g., nebulization of a liquid to vapor) then consideration should be given to both the leachables acquired during storage from the container of the liquid and those acquired during use of the prescribed delivery device. In addition, typically only the final packaged product is evaluated for leachables; however, there may be cases in which an intermediate (e.g., bulk capsule for an inhalation powder) is stored for long periods of time in different primary packaging (e.g., foil pouch) from which compounds may leach. If these compounds that migrate from bulk packaging persist through the drug product's manufacturing process and are entrained in the finished drug product, then they are properly treated as leachables.

The nature of any contact that the packaging and delivery system has with the patient must also be considered. If the contact is only surface contact, then the likelihood of direct chemical migration to the patient is much less than if the contact is with mucosa, tissue, bone, or dentin. The various contact categories are described in *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩ or ISO 10993 (*9*).

LEACHABLES CHARACTERIZATION

The primary goal of any leachables study is leachables characterization; i.e., the discovery, identification, and quantitation of leachables present in a particular drug product. Analytical methods for leachables characterization are developed based on the nature of the drug product matrix, the identities and possible accumulation levels of potential leachables, and the required sensitivity based on an adopted leachables evaluation threshold and the capabilities of the analytical methods employed. Unlike a typical drug product impurity method where target analytes are related to the drug substance, leachables have a wide chemical diversity and can come from various sources in the packaging/delivery system. Leachables also have a wide range of possible accumulation levels in a drug product. Taken in total, these factors present a significant challenge for trace analysis, especially in the case of organic leachables identification. Under certain circumstances, this challenge can be mitigated by performing the process of potential leachables identification outside of the leachables assessment, for example via extractables assessment in simulated extractables studies (see *Simulation Studies*).

Before describing the processes, analytical techniques, and methods involved in leachables characterization, it is appropriate to state that the ultimate objective of thorough leachables characterization as defined above cannot be realized in all cases, even when state-of-the-art analytical chemistry is practiced with best available skill and diligence. It is a reality that there is no analytical technique or combination of analytical techniques that is capable of the discovery, identification, and quantitation of any and all organic and inorganic leachables. For example, authentic reference compounds for organic leachables may not be available in all cases for confirmation of identifications or for quantitative instrument calibration. Given these circumstances, the practical objective of leachables characterization must therefore be the discovery, identification, and quantitation of individual leachables present in a drug product above a predetermined level, or "threshold", to a reasonable degree of scientific certainty and exercised with appropriate due diligence.

Analytical Thresholds

The starting action in leachables method development is to establish the level at which the method must perform at to accomplish the appropriate leachables characterization functions. This level is known as the analytical threshold. Minimally, an appropriate method must function at all levels greater than or equal to the analytical threshold. As discussed previously, such an analytical threshold can be based on various criteria, including safety considerations. An example of a safety-based threshold is the SCT as established for OINDP. In order to define the SCT in terms that facilitate laboratory analysis, it must be converted from units of exposure (i.e., µg/day) to units of concentration (e.g., µg/mL, µg/g, µg/canister, µg/vial, etc.). This is accomplished by considering the dose parameters for a given drug product per the drug product's label claim. The resulting analytically useful threshold is termed the Analytical Evaluation Threshold (AET) (*2*). Previously characterized target leachables will have known safety profiles and previously established leachables thresholds. In any event, thresholds can be used for the basis for analytical method development unless other product considerations, such as compatibility, dictate a lower level is necessary.

A general formula for converting the SCT (0.15 µg/day) to an AET is as follows:

$$\mathrm{AET}\left(\frac{\mu g}{\text{container}}\right) = \left(\frac{0.15 \mu g/\text{day}}{\text{doses/day}}\right) \times \left(\frac{\text{labeled doses}}{\text{container}}\right)$$

Further, for liquid dosage forms:

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$$\text{AET}\left(\frac{\mu g}{mL}\right) = \frac{\mu g}{\text{container}} \div \frac{mL}{\text{container}}$$

Further, for solid dosage forms:

$$\text{AET}\left(\frac{\mu g}{g}\right) = \frac{\mu g}{\text{container}} \div \frac{g}{\text{container}}$$

This AET establishes the level at which unknown leachables should be identified and quantified in a particular drug product, and can therefore be used as a basis for analytical method development.

Analytical Method Requirements

Analytical method requirements for leachables characterization are based on the determined AET (or an alternative valid threshold concept), and information on potential leachables obtained from extractables assessments of packaging components and materials, including information from component/material suppliers. Since leachables are typically a subset of extractables or chemically linked to extractables, it may be the case that analytical methods used for leachables characterization can be based on those used for extractables characterization (see ⟨1663⟩). Any analytical method for leachables that is used for drug product stability studies in support of product registration, establishing leachables–extractables correlations for high-risk dosage forms, or the development of leachables specifications and acceptance criteria must be subject to complete validation using industry-accepted validation practices.

Preparing the Drug Product for Analysis—Sample Preparation

Sample preparation for leachables characterization is a function of the chemical nature of the potential leachables, the chemical nature of the drug product sample matrix, and the analytical technique(s) to be applied. The drug product matrix can present a significant challenge for organic leachables characterization. Drug product matrices contain the active pharmaceutical ingredient and excipients, which are typically present at high levels relative to leachables (except in certain high potency drug products). Analytical methods for organic leachables usually incorporate sample preparation procedures to separate leachables from the drug product matrix and concentrate them for analysis. The exact details of sample preparation procedures are unique to the individual drug product and while it is impossible to anticipate every scenario, the following general statements can be made:

- *Aqueous dosage form* (e.g., inhalation solutions, small and large volume parenterals, ophthalmic solutions, etc.) leachables can be recovered using liquid-liquid extraction with water immiscible organic solvents, such as dichloromethane, hexane or petroleum ether, etc. The pH of the aqueous sample can be manipulated (i.e., raised or lowered) in order to enhance extraction of weakly acidic or basic leachables, or reduce extraction problems caused by the relatively high concentration of active pharmaceutical ingredient and excipients. The resulting organic extract can be dried if required (e.g., with magnesium sulfate drying agent) and concentrated if required by techniques that remove the solvent, such as evaporation with a gentle stream of dry nitrogen, rotary evaporation, or a Kuderna-Danish concentrator, etc. Concentrated organic extracts can be analyzed directly by GC-based methods; however, for HPLC-based methods using aqueous mobile phases the organic extract can be reduced to dryness (or near dryness) and the resulting residue of leachables taken up in a water miscible solvent (e.g., acetonitrile, methanol, etc.). Volatile leachables (e.g., solvents) can be analyzed directly from aqueous drug product samples with GC combined with headspace sampling. Note that recoveries of certain leachables can be affected by extraction and extract concentration procedures.
- *Solid dosage form* (e.g., solid orals, inhalation powders, lyophilized powders, etc.) leachables can be recovered (for example) by dissolving the drug product with an aqueous solution and applying liquid-liquid extraction and extract concentration, as above. Headspace sampling and GC analysis of volatile leachables can be accomplished on the aqueous samples or, in some cases, directly on the solid dosage form. It is also possible to dissolve the drug product sample in another appropriate and analytically expedient medium (e.g., an organic solvent) for direct analysis by GC; however it is possible that matrix effects and interferences from the active ingredients and excipients could result.
- *Oral liquid dosage form* leachables can be recovered by diluting the drug product sample in aqueous solution and applying liquid-liquid extraction and extract concentration, as above. Headspace sampling and GC analysis of volatile leachables can be accomplished on the aqueous samples or, in some cases, directly on the oral liquid dosage form. It is also possible to dissolve the drug product sample in another appropriate and analytically expedient medium (e.g., an organic solvent) for direct analysis by GC; however, it is possible that matrix effects and interferences from the active ingredients and excipients could result.
- *Cream and ointment dosage form* leachables can be recovered by dissolving the drug product sample in an aqueous solution, filtering, and applying liquid-liquid extraction and extract concentration, as above. Headspace sampling and GC analysis of volatile leachables can be accomplished on the aqueous samples. It is also possible to dissolve the drug product sample in another appropriate and analytically expedient medium (e.g., an organic solvent) for direct analysis by GC; however, it is possible that matrix effects and interferences from the active ingredients and excipients could result.

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- Dosage forms with nonaqueous drug product vehicles (e.g., metered dose inhalers with organic solvent propellants) require special sample preparation procedures, which are discussed in ⟨1664.1⟩.

Development of sample preparation methods for leachables characterization can be accomplished through the use of appropriate test samples, such as accelerated drug product samples that have been aged under accelerated conditions (e.g., 40°/75%RH on 3-month storage), drug product or placebo samples spiked with known potential leachables, and/or a simulated drug product matrix spiked with known potential leachables. Recoveries of spiked potential leachables should be assessed and optimized during method development. Internal standards can be included to improve quantitative accuracy and precision.

Note that the sample preparation for leachables characterization must create a test sample in a form amenable to the analytical technique to be applied, and appropriately concentrated so that individual leachables can be characterized relative to the selected threshold.

Analytical Techniques

Analytical techniques applied to leachables characterization are the same as those applied to extractables characterization, which are summarized and discussed in ⟨1663⟩. Scouting analyses in general are not applied to leachables characterization, as the drug formulation may interfere with the scouting methods (see ⟨1663⟩). The most useful analytical techniques for discovery, identification (either by qualitative or structural analysis), and quantitation of organic leachables are those that combine GC and HPLC with mass spectrometry [i.e., GC/MS and HPLC (or LC)/MS]. Headspace sampling can also be interfaced with GC/MS to address volatile compounds. Other detection systems for both GC and HPLC that are not compound specific (e.g., FID, UV, etc.) are potentially useful for leachables discovery and quantitation, but not in general for identification. The combination of GC and HPLC techniques has the sensitivity and specificity required to characterize the diversity of chemical compounds found in many leachables samples. Analytical methods for leachables should be capable of characterizing target as well as new (or unspecified) leachables (e.g., scanning GC/MS or LC/MS); however, when additional sensitivity is required due to the use of analytically challenging thresholds, dedicated target compound methods (e.g., GC/MS with selected ion monitoring) can be used. It is also possible, with appropriate validation, to use methods based on techniques that are not compound specific (i.e., GC/FID, HPLC/UV, etc.).

Structural analysis of leachables should be accomplished with a systematic process identical to that described in ⟨1663⟩ for extractables, and to a level of confidence sufficient for safety assessment. A discussion of the principles of both gas and liquid chromatography is available in *Chromatography* ⟨621⟩. A discussion of the principles of mass spectrometry (including both GC/MS and LC/MS) is available in *Mass Spectrometry* ⟨736⟩. Although chromatographic-based hybrid analytical techniques are most commonly applied to leachables characterization, other analytical techniques with compound-specific detection capability (e.g., nuclear magnetic resonance spectroscopy) can be employed.

Quantitative Methods—Validation Considerations

Validation of quantitative leachables methods should be accomplished according to industry accepted practices, criteria, and standards, such as *Validation of Compendial Procedures* ⟨1225⟩ and (*10*). The extent of validation required depends on the goals of the leachables study in which the analytical method is being utilized. Validation parameters may include: accuracy, precision (repeatability, intermediate precision), specificity, limit-of-detection, limit-of-quantitation, linearity and range, and robustness. System suitability tests and criteria should also be developed for each leachables method. Special considerations for individual validation parameters relative to leachables methods are as follows:

- *Accuracy and precision*—The validation parameters of accuracy and precision (repeatability and intermediate precision) are typically evaluated using drug product samples spiked with known amounts of target leachables. The choice of drug product spiking matrix used for these evaluations should be one that has had little-to-no contact with the packaging materials used in the final drug product, and therefore little-to-no measurable levels of endogenous leachables. Suitable spiking matrices can include freshly manufactured drug product and simulated drug product vehicles. Spiking levels should be determined based on results from accelerated stability studies or estimated from the known amounts of potential target leachables determined from extraction studies. Accuracy is typically performed at three spiking levels, which can also be determined based on results from accelerated stability studies or estimated from the known amounts of potential target leachables determined from extraction studies.
- *Linearity and range*—Since potential leachables are present in packaging components at widely varying levels, actual drug product leachables can likewise appear at widely varying levels. The best accuracy and precision are achieved when the validated linear range considers the potential maximum accumulation levels of each target leachable or chemical class of leachables.
- *Limit of detection/Limit of quantitation*—To detect and quantitate unknown leachables, the limit of quantitation should be at or below the designated analytical threshold (e.g., AET).
- *Specificity*—Evaluation of method specificity can be accomplished by evaluating chromatographic peak purity in spiked and nonspiked drug product samples. For GC-based quantitative methods, this can be accomplished by GC/MS. For HPLC-based methods, either LC/MS or LC/DAD (diode array detection) can be used. Specificity can be qualitatively demonstrated if there are no observable method interferences related to the chemical entities present in the drug product.
- *Robustness*—A design-of-experiments statistical approach with consideration of critical analytical method parameters (e.g., HPLC flow-rate, HPLC column, mobile phase gradient, etc.) should be used to create robustness evaluation protocols. Other approaches, such as serial change of critical parameters, can also be applied.
- *System suitability*—Chromatography-based analytical methods, such as those described in ⟨621⟩, should include appropriate system suitability criteria for routine method evaluation, including tests for method linearity, precision, sensitivity, and specificity as appropriate. These parameters should be evaluated with an appropriately constituted test mixture(s) each time the quantitative leachables method is used, and should include appropriate system suitability

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acceptance criteria based on the method validation results. For example, sensitivity may be confirmed by analysis of standards prepared at the analytical threshold.

Several examples of validated leachables methods from the scientific literature have been documented in the chemical literature (*11–16*).

ESTABLISHING A LEACHABLES–EXTRACTABLES CORRELATION

A leachables–extractables correlation is established when actual drug product leachables can be linked either qualitatively or quantitatively with extractables from corresponding extractables assessments of individual materials of construction, packaging components, or packing systems. Leachables–extractables correlations are important for several reasons, including justifying the use of routine extractables release tests of packaging components as an alternative to leachables testing during stability studies for high-risk drug products, establishing the source of a leachable producing an OOS result for a low-risk drug product, change control, and ongoing quality control, etc.

A *qualitative correlation* is demonstrated when a leachable is linked either *directly* or *indirectly* to an extractable (i.e., potential leachable). For example, hexadecanoic acid observed in a leachables profile can be directly linked with hexadecanoic acid observed in the extractables profiles of one or more primary packaging components. The ethyl ester of hexadecanoic acid observed in the same leachables profile can be indirectly linked with hexadecanoic acid observed in one or more extractables profiles, if ethanol is a known drug product formulation constituent and it is shown that an esterification reaction can occur in the drug product during storage. For an appropriate quantitative leachables–extractables correlation to exist, the quantity of any individual leachable over the shelf-life of a drug product must be mathematically related to the quantity of the corresponding extractable in its source. One of the more simple mathematical relationships between an extractable and a leachable is that the quantity of the leachable in the drug product should be less than or equal to the quantity of the corresponding extractable. For example, the concentration of butylatedhydroxytoluene (BHT) present in a drug product formulation was determined to be 5 µg/mL. BHT was extracted from a primary packaging system component at a level of 300 µg/component. If the drug product packaging system incorporates one of these components per dosage form and the packaged formulation has a volume of 50 mL, then a quantitative leachables–extractables correlation is established, as BHT was extracted in the amount of 300 µg (300 µg/component × 1 component) and was leached in the amount of 250 µg (5 µg/mL × 50 mL). As a result, it can be concluded that on the average 50 µg of BHT is unaccounted for (300 µg extracted – 250 µg leached), and that this quantity was either not leached from the packaging component into the formulation (more likely) or lost by some other process (less likely).

For high-risk drug products, leachables–extractables correlations may be established over multiple batches of drug product (accelerated or at end of shelf-life) and multiple batches of packaging components. Extractables studies should ideally be conducted on the same lots of components that were used to manufacture the drug product batches used in primary stability studies (and therefore on the drug product batches on which leachables testing was conducted to establish leachables–extractables correlations).

If the maximum level of any specific leachable in the formulation during stability studies was substantially greater than the calculated maximum potential accumulation levels of that same leachable as established by the extraction study, and the extraction studies were conducted on the same lots of components used to make the primary drug product stability batches, it can be concluded that the extraction study was incomplete and therefore a leachables–extractables correlation for that specific leachable cannot be established. In this case, either the extraction study can be augmented with experiments that produce an extractable level exceeding the maximum level of the leachable, or the leachable can be controlled via the drug product specifications for shelf-life stability testing, and release testing as an extractable at the component level is inadequate to control this leachable.

If a leachable–extractables correlation cannot be established, possible explanations include: inadequate extractables assessments of packaging components (see ⟨1663⟩); unreported changes in packaging component composition or manufacturing processes; unreported changes in the identity of packaging components.

CONSIDERATIONS IN DEVELOPING LEACHABLES SPECIFICATIONS AND ACCEPTANCE CRITERIA

The validated analytical methods and information obtained from those methods in a drug product leachables study can be used to develop drug product leachables specifications and acceptance criteria (i.e., limits). In certain circumstances, most commonly encountered with high-risk dosage forms (such as OINDP), it may be meaningful, useful, and at times required to routinely monitor finished drug products for leachables. Under such circumstances, leachables specifications and acceptance criteria must be established. One means by which such specifications and acceptance criteria could be developed includes testing a minimum of three drug product batches to determine their leachables levels. After thorough chemical and safety evaluation, the test data from the three or more batches can be used to establish acceptance criteria for targeted leachables, consistent with 1) the qualitative and/or quantitative results of leachables studies, 2) a consideration of the capabilities of the drug product's manufacturing process, and 3) a consideration of the potential safety, compatibility, and/or drug product quality impact of the leachables. It is important to note that leachables specifications should be applicable to a product during all stages of its shelf-life, including release and at end of shelf-life. This is the case since leachables accumulate over the entire shelf-life of a drug product.

When a change occurs in a product for which leachables specifications and acceptance criteria have been established, it is important to review the analytical method and re-evaluate the acceptance criteria and make adjustments to the specifications and acceptance criteria as appropriate and scientifically justified. A change in components that results in an increase in leachables concentrations beyond the levels qualified will necessitate the toxicological evaluation of the proposed levels, as would be the case for any impurity.

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Acceptance criteria can be both qualitative and quantitative for both known and unspecified leachables. For example, a typical leachables specification could include:

- Quantitative end of shelf-life limits for target leachables, which apply over the shelf-life of the drug product
- A quantitative limit for "unspecified" (i.e., previously unidentified and uncorrelated) leachables (identification of unspecified leachables is required for accurate quantitation and toxicological evaluation).

ADDITIONAL CONSIDERATIONS

Simulation Studies

Occasions may arise in which it is not analytically feasible (due to challenging thresholds, for example) to successfully discover and identify all actual leachables in a drug product leachables study. This circumstance can be managed if the activities of discovery and identification of probable leachables are accomplished in an extraction study, where samples and analyte concentrations are more easily manipulated to achieve the necessary analytical performance. In such a circumstance, the actual drug product leachables assessment is simplified to a high-sensitivity quantitation of targeted leachables that have been discovered and identified as part of this extraction study.

In order to facilitate the discovery and identification of probable leachables, the extraction study must be similar in design to a drug product leachables study. Such an extraction study seeks to simulate the circumstances experienced by the drug product but should produce a test sample that is easier to characterize than the drug product itself. For such a study to be relevant in establishing appropriate target leachables, the solvent(s) used to generate the test sample must have nearly the identical propensity to leach as the drug product formulation. Such a study should be accelerated versus the leachables study so that the extractables, reflecting potential target leachables, can be discovered and identified in a timely fashion. Differences in the study design between this simulation study and the drug product leachables study are: 1) that the drug product formulation has been replaced with a simulating solvent that mimics the formulation; 2) that the conditions of contact have been accelerated, so as to increase both the concentrations of probable leachables and the rates of migration of probable leachables into the simulating solvent; and 3) that the test article can be either the complete packaging and delivery system or separate components of that system. Factors to consider in designing and justifying the simulating solvent(s), along with recommendations on the analytical approach used to characterize the simulating extract for extractables as potential target leachables are discussed in ⟨1663⟩. Given the intent of the simulation study, which is the discovery and identification of extractables as target leachables, simulation studies must also be driven by relevant thresholds.

It is possible that in cases of very low thresholds (e.g., AETs), quantitation of drug product leachables might still not be analytically feasible, even with high sensitivity target compound analytical methods. In such cases, the results of the simulation study (probable leachables identities and concentrations) may be sufficient to establish patient safety and the quality impact of the actual drug product leachables. **To the extent that the simulation study mimics the drug product leachables study**, the potential safety or quality impact of a compound as an extractable is an estimate of the potential safety or quality impact of the compound as an actual leachable. If it can be established that a compound quantitated as an extractable under these conditions has an acceptably small impact on safety and quality, then it follows that the same compound as a leachable in the drug product formulation may be assumed to have a similarly low impact on safety and quality as a leachable in the drug product formulation. The acceptability of this approach for any particular drug product needs to be scientifically justified by the drug product applicant.

If a compound is measured as an extractable in a simulation study and targeted as a leachable in a drug product leachables study, the extractables and leachables data for that compound become the basis upon which a leachables–extractables correlation can be made. For such a correlation to be considered to be valid, it is necessary that each leachable concentration in the drug product be less than or equal to the corresponding extractable concentration in the simulated extract, accounting for the uncertainty in the analytical measurements and any justifiable "exaggeration factors" that may have been utilized in the simulation study. Note that in certain justifiable circumstances drug product placebo batches may be used as test articles in drug product leachables studies (stability studies). However, there are also circumstances when placebo batches are not acceptable, such as when there is significant reason to believe that leachables might have an adverse effect on an active pharmaceutical ingredient (e.g., therapeutic proteins).

Inorganic (Elemental) Leachables

The topic of leachables as elemental impurities in pharmaceuticals can be addressed within the overall context of elemental impurities in drug products (e.g., *Elemental Impurities—Limits* ⟨232⟩. Elemental impurities leached from packaging or delivery systems represent only one source of elemental impurities in a drug product, and thus testing a drug product for elemental impurities does not establish that the impurities are leachables.

Testing of the plastic packaging systems and their materials of construction will establish those extractable elemental impurities that are relevant to a particular packaging system, and it may be appropriate to quantify such elemental impurities as leachables in the drug product. Therefore, the results of testing plastic packaging systems should be used to establish those elemental impurities that should be monitored as targeted elemental leachables in the drug product.

In general, guidelines and recommendations about elemental impurities in drug products address safety concerns associated with the elemental impurities. However, it is proper to consider elemental leachables from the broader perspective of the overall quality of the drug product. Thus the process of evaluating elemental leachables may include both the aspects of user safety and product quality.

Note that one of the differences between the testing for organic leachables (and extractables) and for elemental impurities is the nature of the information generated. The testing for organic leachables is based on having established the identity of the chemical compound that is the leachable. Alternatively, the test methods most commonly employed to address elemental

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impurities (atomic spectroscopy) do not establish the compound, or form, that the detected element is present in. For example, sulfur might be present in the form of elemental sulfur (S_8), as the sulfate (SO_4^{-2}), or as a sulfur-containing organic compound (e.g., 2-mercaptobenzothiazole). Also, silicon might be present either as silicone oil or as silicon dioxide (SiO_2). As the form of the elemental impurity may have a marked effect on the impurity's impact on product quality or safety, it may be the case that testing beyond elemental impurity profiling may be necessary to establish the exact chemical form of the elemental impurity and thus ascertain its potential safety or product impact.

An important issue to consider in testing drug products for elemental leachables is establishing which elements to measure as leachables. Considering this, it is noted that testing of the plastic packaging systems and their materials of construction will establish those extractable elemental impurities that are relevant to a specific packaging system. Therefore, the results of testing plastic packaging systems should be used as one means of establishing those elemental impurities that should be monitored as targeted elemental leachables.

SUMMARY

The requirement for, and completeness of, a leachables assessment for any particular drug product can only be determined by the drug product applicant with reference to appropriate regulatory guidance documents. Detailed recommendations for OINDP are presented in ⟨1664.1⟩.

Reference is also made to other compendial chapters in this Pharmacopeia that describe related extraction studies:

1. *Biological Reactivity Tests, In Vitro* ⟨87⟩
2. *Biological Reactivity Tests, In Vivo* ⟨88⟩
3. *Elastomeric Closures for Injection* ⟨381⟩
4. *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩
5. *Plastic Materials of Construction* ⟨661.1⟩
6. *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩

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⟨1664.1⟩ ORALLY INHALED AND NASAL DRUG PRODUCTS

INTRODUCTION

This section addresses specific considerations for leachables in orally inhaled and nasal drug products (OINDP), including metered dose inhalers (MDIs); nasal sprays; inhalation solutions, suspensions, and sprays; and dry powder inhalers (DPIs). Although OINDP can be a combination of products that are comprised of drug and device constituent parts, the primary mode of action is typically through the drug. For this reason OINDP are treated as drugs from a regulatory perspective. Regulatory guidance documents and detailed best practice recommendations specific to OINDP are available (*1–4*). Note that the following discussion is primarily devoted to organic leachables. For consideration of inorganic (i.e., elemental) leachables, see *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

KEY TERMS

In addition to the key terms listed in ⟨1664⟩, some additional key terms more specific to OINDP are the following:

- *Critical components* are packaging components that contact either the drug product formulation or the patient, or that affect the mechanics of the overall performance of the packaging and delivery system, including any necessary secondary packaging. The identification of critical components for a particular OINDP dosage form is the responsibility of the applicant in consultation with appropriate regulatory authorities.
- *Special case compounds* are individual (or classes of) compounds that have special safety or historical concerns as drug product leachables in OINDP, and therefore must be evaluated and controlled as leachables (and extractables) by specific analytical techniques and technology-defined thresholds.

Additional terminology and associated definitions specific to OINDP are available in the cited references (*1–4*).

LEACHABLES ASSESSMENT RATIONALE FOR ORALLY INHALED AND NASAL DRUG PRODUCTS

OINDP are generally categorized as high-risk dosage forms due to safety considerations related to the route of administration and high probability of packaging component interaction with the formulation (see *Table 1* in ⟨1664⟩). The packaging systems used in these drug products incorporate components of various types, including components composed of polymeric (plastic or elastomeric) raw materials with complex chemical compositions and therefore a variety of potential leachables. Chemical entities may migrate (i.e., leach) into the formulation when there is direct contact with the primary packaging and delivery components for extended periods of time. In certain cases, there is also the potential for leaching from secondary and tertiary packaging. In addition, for OINDP, contact of the delivery device with mucosal tissue (mouth or nasal) is generally expected. Leachables studies for some OINDP may be considered separately for packaging components that are in continuous contact with the formulation (e.g., vials, bottles, blisters, metering valve components) versus those that are only in transient contact (e.g., DPI mouthpiece, MDI mouthpiece).

OINDP typically require:

- A leachables stability study for drug product registration that supports intended storage and use conditions throughout the proposed shelf-life (see *Table 1*), ideally on primary drug product stability batches manufactured with the same lots of packaging components used in extraction studies (in order to facilitate a leachables–extractables correlation)
- Sensitive, selective, and fully validated leachables analytical methods
- Leachables assessments based on safety thresholds [Safety Concern Threshold (SCT): 0.15 µg/day, and Qualification Threshold (QT): 5 µg/day total daily intake (TDI) for an individual organic leachable; however, for exceptions see *Special Case Compounds*]
- Complete qualitative and quantitative leachables–extractables correlations (which require that extractables assessments be accomplished on all critical packaging components; see *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩)
- Leachables specifications including acceptance criteria (assumes a complete extractables assessment for each critical packaging component). (Note that in many cases routine extractables testing for release of critical components can be used to control drug product leachables in lieu of routine drug product leachables testing, providing that a comprehensive leachables–extractables correlation is established.)

For OINDP dosage forms based on formulations with relatively lower leaching potential for organic compounds (e.g., aqueous formulations, dry powder formulations), the above requirements should be considered and evaluated on a case-by-case basis, including consultations with the appropriate regulatory authorities.

Table 1. Example Stability Storage Conditions and Testing Time Points for an OINDP Registration Leachables Study (*4*)

Condition (temperature/relative humidity)	Time Points (months)
25 ± 2°C/60 ± 5%RH	3, 6, 12, 18, 24, 36 (to end of shelf-life)
30 ± 2°C/65 ± 5%RH	3, 6, 12, 18, 24, 36 (to end of shelf-life)
40 ± 2°C/75 ± 5%RH	3, 6

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ORALLY INHALED AND NASAL DRUG PRODUCTS DOSAGE FORM TYPES

Metered Dose Inhaler

MDIs or pressurized MDIs (pMDIs) are defined as "drug products that contain active ingredient(s) dissolved or suspended in a propellant, a mixture of propellants, or a mixture of solvent(s), propellant(s), and/or other excipients in compact pressurized aerosol dispensers" (*2,4*). Typical MDIs include a metal canister (stainless steel or aluminum; coated or uncoated), a fixed-volume metering valve (with plastic or elastomeric components), elastomeric seals, and a plastic actuator or mouthpiece (see *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩). MDIs are multidose drug container closure and delivery systems that can contain sufficient formulation for up to several hundred actuations (label claim) per container. Because many of the critical packaging components are in continuous contact with an organic solvent-based formulation, the MDI has the highest risk for formulation-packaging component interaction, and therefore the highest risk for leachables, of all OINDP dosage forms (or any other dosage form). Because of the leaching potential of their organic solvent-based formulations, MDIs would typically be expected to show complete qualitative and quantitative leachables–extractables correlations. Leachables in MDIs should be characterized (i.e., identified and quantitated) at levels above a calculated analytical evaluation threshold (AET). An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an MDI follows.

Given an MDI drug product with 200 labeled actuations per canister, a maximum recommended patient exposure of 12 actuations per day, and a critical valve component mass per valve of 200 mg, for an individual organic leachable derived from this valve component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{12\ \text{actuations/day}}\right) \times (200\ \textit{labeled}\ \text{actuations/canister})$$

$$\text{Leachables Estimated AET} = 2.5\ \mu\text{g/canister}$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular valve component (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (2.5\ \mu\text{g/Canister}) \times \left(\frac{1\ \text{canister/valve}}{0.2\ \text{g elastomer/valve}}\right)$$

$$\text{Extractables Estimated AET} = 12.5\ \mu\text{g/g}$$

The AET calculation should not be modified to account for variables, such as manufacturing overfill in the canister to compensate for leak rate or fill variability, unless such modification can be scientifically justified.

Analytical methods for leachables testing of MDI drug products can be based on processes such as "cold filtration" of suspension formulations to remove active ingredient and excipient particles (*5*) or careful venting of the volatile organic propellant, which retains leachables in a residue within the canister (*6*). Because sample preparation procedures for MDI formulations can be complex and typically require the volatile propellant to be reduced to dryness at some point, creating the possibility for loss of leachables before sample analysis, it is particularly important to demonstrate adequate recoveries of leachables through the use of spiked MDI samples.

Although it is unlikely to contribute leachables to the emitted drug product aerosol plume, potential patient exposure to chemical entities from the MDI plastic actuator or mouthpiece should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies and references required to assess patient exposure to actuator- or mouthpiece-derived chemicals include reference to indirect food additive regulations and the application of *Biological Reactivity Tests, In Vitro* ⟨87⟩ and *Biological Reactivity Tests, In Vivo* ⟨88⟩ (*2*). Note that "spacers" and other devices designed for use with MDIs should also be characterized if a particular device is specified on the drug product label.

When constructed from materials acceptable for food contact, MDI actuators and mouthpieces, "spacers", and other components and devices specified in the drug product labeling generally only require appropriate characterization (i.e., extraction studies and routine extractables testing) in order to assure continued consistent composition of the component or device.

In addition, based on applicable regulatory guidance (*2*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of surface organic residue release tests for incoming uncoated metal canisters, with appropriate acceptance criteria
- Development and validation of extractables release tests for the inner surfaces of incoming coated canisters, with appropriate acceptance criteria
- Development and validation of extractables release tests for incoming metering valve critical components, with appropriate acceptance criteria
- Development and validation of extractables profile release tests for incoming actuators or mouthpieces, with appropriate qualitative and quantitative acceptance criteria.

Nasal Sprays

Nasal sprays are defined as "drug products that contain active ingredients dissolved or suspended in a formulation, typically aqueous-based, which can contain other excipients and are intended for use by nasal inhalation" (*1,4*). Nasal sprays include a

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plastic container and components (usually plastic) that are responsible for formulation metering, atomization, and delivery to the patient (see ⟨601⟩). Critical components include those that are in constant contact with the formulation (e.g., the container, dip tube) and components that are in the liquid pathway during actuation of the device and that do not permit quick evaporation of residual surface liquid (*3*). Because nasal sprays are typically aqueous-based formulations, and the vast majority of potential organic leachables are relatively lipophilic, the risk for formulation-packaging component interaction is lower relative to the organic propellant-based MDIs, and the risk for organic leachables is lower. Leachables in nasal sprays should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for a nasal spray follows.

Given a nasal spray drug product with 120 labeled actuations per container, a maximum recommended patient exposure of 4 actuations per day, and a critical component (plastic dip tube) mass of 250 mg, for an individual organic leachable derived from this component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{4\ \text{actuations/day}}\right) \times (120\ \textit{labeled}\ \text{actuations/container})$$

$$\text{Leachables Estimated AET} = 4.5\ \mu\text{g/container}$$

Given a total fill volume of 10 mL:

$$\text{Estimated AET} = (4.5\ \mu\text{g/container})/(10\ \text{mL/container})$$

$$\text{Estimated AET} = 0.45\ \mu\text{g/mL}$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular plastic dip tube (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (4.5\ \mu\text{g/container}) \times \left(\frac{1\ \text{container}}{0.25\ \text{g material/tube}}\right)$$

$$\text{Extractables Estimated AET} = 18\ \mu\text{g/g}$$

The AET calculation should not be modified to account for variables, such as manufacturing overfill, unless such modification can be scientifically justified.

All nasal spray packaging system critical components should be subjected to extractables assessments (see ⟨1663⟩). Potential patient exposure to chemical entities from nasal spray critical components not in continuous contact with the drug product formulation should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies and references required to assess patient exposure to nonformulation contact critical component derived chemicals include reference to indirect food additive regulations and the application of ⟨87⟩ and ⟨88⟩ (*1*).

When constructed from materials acceptable for food contact, nasal spray critical components not in continuous contact with the drug product formulation generally only need to be appropriately characterized (i.e., extraction studies and routine extractables testing) in order to assure continued consistent composition of the component.

In addition, based on applicable regulatory guidance (*1*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of extractables release tests for incoming container closure and pump critical components, with appropriate qualitative and quantitative acceptance criteria.

Inhalation Solutions, Suspensions, and Sprays

Inhalation solutions, suspensions, and sprays are defined as "drug products that contain active ingredients dissolved or suspended in a formulation, typically aqueous-based, which can contain other excipients and are intended for use by oral inhalation" (*1,4*). Inhalation solutions and suspensions are intended for use with a nebulizer (*1,4*). Inhalation sprays, like MDIs and nasal sprays, are combination products where the components responsible for the metering, atomization, and delivery of the formulation to the patient are a part of the container closure system (*1,4*). Critical components include components that are in constant contact with the formulation and components that are in the liquid pathway during actuation of the device and that do not permit quick evaporation of residual surface liquid. Leachables in inhalation sprays should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an inhalation spray follows.

Given an inhalation spray drug product with 120 labeled actuations per container, a maximum recommended patient exposure of 4 actuations per day, and a critical component (plastic dip tube) mass of 400 mg, for an individual organic leachable derived from this component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{4\ \text{actuations/day}}\right) \times (120\ \textit{labeled}\ \text{actuations/container})$$

$$\text{Leachables Estimated AET} = 4.5\ \mu\text{g/container}$$

Given a total fill volume of 4.5 mL:

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Estimated AET = (4.5 µg/container)/(4.5 mL/container)

Estimated AET = 1 µg/mL

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular plastic dip tube (see ⟨1663⟩):

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{4\ \text{actuations/day}}\right) \times (120\ \textit{labeled}\ \text{actuations/container})$$

Extractables Estimated AET = 11.3 µg/g

The AET calculation should not be modified to account for variables, such as manufacturing overfill, unless such modification can be scientifically justified.

Because inhalation solutions and suspensions are similar to nasal spray and inhalation spray drug products in that they are typically aqueous-based formulations, and the vast majority of potential organic leachables are relatively lipophilic, the risk for formulation-packaging component interaction is lower relative to the organic propellant-based MDIs, and the risk for organic leachables is lower. However, unlike MDIs, nasal and inhalation spray drug products, inhalation solutions, and suspensions are typically packaged in plastic unit dose containers (i.e., nebules). Leaching can potentially occur from the unit dose container [e.g., low-density polyethylene (LDPE)], which is in long-term continuous contact with the drug product formulation. It is also possible that organic chemical entities associated with paper labels, adhesives, inks, etc. in direct contact with the permeable unit dose container can migrate through the container and into the formulation. Leachables from tertiary packaging systems (e.g., cardboard shipping containers) are also possible. Leachables in inhalation solutions and suspensions should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an inhalation solution follows.

Given an inhalation solution with 3 mL of drug product contained in a LDPE unit dose vial (1 g total weight of LDPE), with a maximum recommended patient exposure of three vials per day, for an individual organic leachable derived from this component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{3\ \text{doses/day}}\right) \times (1\ \textit{labeled}\ \text{dose/container})$$

Leachables Estimated AET = 0.05 µg/container

Estimated AET = (0.05 µg/container)/(3 mL/container)

Estimated AET = 0.017 µg/mL

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular plastic unit dose vial (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (0.05\ \mu\text{g/container}) \times \left(\frac{1\ \text{container}}{1\ \text{g material/container}}\right)$$

Extractables Estimated AET = 0.05 µg/g

The challenge of characterizing drug product leachables at levels of 17 ng/mL in an aqueous drug product is considerable, even given the capabilities of modern analytical chemistry. For this particular inhalation solution example, it might be appropriate to implement a simulation study (see ⟨1663⟩ and ⟨1664⟩) to facilitate the discovery and identification of probable leachables, with actual drug product leachables being quantitated (if required) with high-sensitivity target compound analytical techniques and methods.

All inhalation solution, suspension, and spray packaging system critical components should be subjected to extractables assessments (see ⟨1663⟩). Potential patient exposure to chemical entities from inhalation solution, suspension, and spray critical components not in continuous contact with the drug product formulation should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies and references required to assess patient exposure to nonformulation contact critical component-derived chemicals include reference to indirect food additive regulations and application of ⟨87⟩ and ⟨88⟩ (*1*). When constructed from materials acceptable for food contact, inhalation solution, suspension, and spray critical components not in continuous contact with the drug product formulation generally only need be appropriately characterized (i.e., extraction studies and routine extractables testing) in order to assure continued consistent composition of the component. Critical components of nebulizers and other devices designed for use with inhalation solutions and suspensions should also be characterized with respect to extractables and leachables if a particular device is specified in the drug product labeling.

Based on applicable regulatory guidance for inhalation solutions, suspensions and sprays (*1*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of extractables release tests for incoming container closure and pump critical components, with appropriate qualitative and quantitative acceptance criteria
- Consideration of validated tests for probable leachables from labels, inks and adhesives, etc., with appropriate acceptance criteria (should these be appropriate and applicable).

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Dry Powder Inhalers and Inhalation Powders

DPIs are defined as "drug products designed to dispense powders for inhalation" (*2,4*). The drug substance in an inhalation powder has a particle size distribution in the respirable range, and may be a physical mixture of active pharmaceutical ingredient(s) with carrier particles or a formulated combination of active ingredient and excipients (see ⟨601⟩). The powder may be contained in a unit dose packaging system (e.g., capsule, blister), or reside in bulk in a reservoir inside the delivery device itself. In the latter case, the dose is metered by the device. The delivery device may actively disperse the powder from the container or rely on patient inspiration to supply the energy necessary to disperse the particles. The components and the design of the device are integral to the aerosol characteristics (i.e., mass and particle size distribution) of the formulation delivered to the patient. There is a wide diversity of DPI designs and characteristics (*2*).

Of all OINDP, the DPI has the lowest risk of exposing a patient to leachables at significant levels. The reasons for this are:

1. The DPI drug product formulation is a dry powder, and contains no solvent, either organic or aqueous, which can promote leaching of organic (or inorganic) chemical entities.
2. In a unit dose DPI, the drug product formulation is contained in a separate packaging system, and is usually only in transient contact with critical components of the device itself.

The most likely source of leachables in a unit dose DPI would be the material composing the unit dose container, such as a foil laminate blister or capsule material, or the material composing the drug product reservoir in a multidose DPI (including antistatic surface additives). Leaching would have to occur either via direct contact of the drug product powder with the packaging material, via volatilization of organic chemical entities from the container closure material with deposition on the dry powder, or via migration of organic chemical entities through the primary packaging material with deposition on the dry powder. The possibility of observing leachables from the DPI unit dose container is best evaluated with detailed extraction studies on the container material to identify potential leachables, which could possibly migrate to the dry powder by either solid-solid contact or volatilization and have potential safety concerns.

The device and packaging materials are typically evaluated for potential leachables by extraction and simulation studies (see ⟨1663⟩) to determine whether there are chemical entities at levels that would pose a safety concern. The evaluation of materials that contain the inhalation powder must consider the inks and any other processing aids used in the manufacture of the container so that all potential leachables are characterized. The types of compounds of greatest concern for inhalation powders are those that may migrate from the primary packaging (i.e., unit dose container or multidose reservoir) into the formulation. Extraction and simulation studies should consider all possible mechanisms of leaching, including volatilization. Actual and potential leachables in inhalation powders derived from critical components of the packaging system or device that may have continuous long-term contact with the drug product formulation should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an inhalation powder follows.

Given a DPI containing 13 mg of inhalation powder in a unit dose blister with 50 mg of blister material either in direct contact with the formulation or capable of volatilizing leachables into the headspace above the formulation, with a maximum recommended daily exposure of 2 actuations per day, for an individual organic leachable derived from this material, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{2\ \text{doses/day}}\right) \times (1\ \textit{labeled}\ \text{dose/blister})$$

$$\text{Leachables Estimated AET} = 0.075\ \mu\text{g/blister}$$

To convert relative to the total mass of drug product in a blister:

$$\text{Estimated AET} = (0.075\ \mu\text{g/blister})/(0.013\ \mu\text{g drug product/blister})$$

$$\text{Estimated AET} = 5.8\ \mu\text{g/g drug product}$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular blister material (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (0.075\ \mu\text{g/blister}) \times \left(\frac{1\ \text{blister}}{0.05\ \text{g material/blister}}\right)$$

$$\text{Extractables Estimated AET} = 1.5\ \mu\text{g/g}$$

The challenge of characterizing drug product leachables at levels of 5.8 µg/g in an inhalation powder is considerable, even given the capabilities of modern analytical chemistry. For this particular DPI example, it might be appropriate to implement a simulation study (see ⟨1663⟩ and ⟨1664⟩) to facilitate the discovery and identification of probable leachables from the blister material, with actual drug product leachables being quantitated (if required) with high-sensitivity target compound analytical techniques and methods.

All inhalation powder packaging system and DPI device critical components should be subjected to extractables assessments (see ⟨1663⟩). Potential patient exposure to chemical entities from inhalation powder packaging system and DPI device critical components not in continuous contact with the drug product formulation should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies might be required to assess patient exposure to nonformulation contact critical component derived chemicals, including reference to food additive regulations and application of ⟨87⟩ and ⟨88⟩ (*2*).

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When constructed from materials acceptable for food contact, inhalation powder packaging system and DPI device critical components not in continuous contact with the drug product formulation generally only need be appropriately characterized (i.e., extraction studies and routine extractables testing) to assure continued consistent composition of the component.

In addition, for DPIs and inhalation powders, and based on applicable regulatory guidance (*2*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of extractables release tests for incoming inhalation powder packaging system and DPI device critical components, with appropriate qualitative and quantitative acceptance criteria.

ADDITIONAL CONSIDERATIONS

Analytical Uncertainty

An AET is that concentration above which unknown leachables should be characterized and reported for toxicological assessment. Target leachables (previously characterized as potential or probable leachables from extractables or simulation studies) will have known safety profiles and previously established leachables thresholds. In addition, reference compounds for previously characterized potential leachables will allow for accurate and precise quantitation of those target leachables as actual drug product leachables. Characterization of unknown leachables requires consideration of analytical uncertainty, as the location of an AET in a given leachables profile (e.g., a gas chromatography/mass spectrometry [GC/MS] chromatogram) must be accomplished relative to an internal standard(s) within the leachables profile. Analytical uncertainty for a particular analytical technique or method can be estimated based on the analysis of a series of reference compounds to create a response factor database. The reference compounds included in this database should represent all known potential leachables (i.e., as determined from extractables assessments). For OINDP, it is recommended (*4*) that the estimated AET be lowered by a factor defined as 1% relative standard deviation in an appropriately constituted response factor database, or a factor of 50% of the estimated AET, whichever is greater. Detailed examples of response factor databases and AET determinations are available (*4*).

Special Case Compounds

Polycyclic Aromatic Hydrocarbons (PAHs) or Polynuclear Aromatics (PNAs), *N*-nitrosamines, and 2-mercaptobenzothiozole (2-MBT) are considered to be "special case" compounds (i.e., compounds with special safety and historical concerns), requiring special characterization studies using specific analytical techniques and methods (*1,2,4*). Thresholds for characterization of these compounds as extractables or leachables in OINDP are typically based on the limits of these specific analytical techniques and methods. *Table 2* lists the PNAs and *N*-nitrosamines that, along with 2-MBT, are typically investigated as extractables and leachables in OINDP.

Table 2. PAHs, PNAs, and *N*-Nitrosamines Typically Investigated as Extractables and Leachables for OINDP

Target PAHs/PNAs	Target *N*-nitrosamines
Naphthalene	*N*-Nitrosodimethylamine
Acenaphthylene	*N*-Nitrosodiethylamine
Acenaphthene	*N*-Nitrosodi-*n*-butylamine
Fluorene	*N*-Nitrosomorpholine
Phenanthrene	*N*-Nitrosopiperidine
Anthracene	*N*-Nitrosopyrrolidine
Fluoranthene	—
Pyrene	—
Benzo(*a*)anthracene	—
Chrysene	—
Benzo(*b*)fluoranthene	—
Benzo(*k*)fluoranthene	—
Benzo(*e*)pyrene	—
Benzo(*a*)pyrene	—
Indeno(123-*cd*)pyrene	—
Dibenzo(*ah*)anthracene	—
Benzo(*ghi*)perylene	—

PNAs have been associated with carbon black filler used in many types of elastomer. Analysis of PNAs, either as elastomer extractables or as drug product leachables, usually involves quantitative extraction followed by highly specific and sensitive analysis of resulting extracts. GC/MS with selected-ion-monitoring has been reported for analysis of target PNAs as leachables in MDI drug products, for example (*5*). *N*-Nitrosamines are reaction products between specific organic precursor molecules, secondary amines (R_2NH), and a "nitrosating agent". In the compounding of rubber, secondary amines are likely formed from

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certain vulcanization accelerators such as thiurams and dithiocarbamates. Potential nitrosating agents include NO^+, N_2O_3, N_2O_4, etc., certain of which can be formed from commonly used chemicals such as sodium nitrite ($NaNO_2$), which has many industrial uses. Analysis of *N*-nitrosamines in rubber as potential leachables involves quantitative extraction followed by analysis of extracts with gas chromatography/thermal energy analysis detection (GC/TEA) (*7*). Analysis of *N*-nitrosamines as leachables in MDI drug products using GC/TEA has been reported (*6*). The 2-MBT is a vulcanization accelerator, which is used in certain sulfur-cured elastomers, and can be analyzed by extraction followed by LC/MS (*4*).

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⟨1724⟩ SEMISOLID DRUG PRODUCTS—PERFORMANCE TESTS

SCOPE

The scope of this general chapter is to provide general information for performance testing of semisolid drug products, various types of equipment employed for such testing, and potential applications of the performance testing.

PURPOSE

This chapter provides general information about performance testing of semisolid drug products, the theory and applications of such testing, information about the availability of appropriate equipment, and likely developments in performance testing of semisolid drug products. General chapter *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩ provides information related to product quality tests for topical and transdermal dosage forms, *Drug Release* ⟨724⟩ provides procedures and details for testing drug release from transdermal systems, and this chapter ⟨1724⟩ provides procedures for determining drug release from semisolid dosage forms.

INTRODUCTION

This chapter provides general information for in vitro testing of semisolid drug products. Semisolid dosage forms include creams, ointments, gels, and lotions. Semisolid dosage forms may be considered extended-release preparations, and their drug release depends largely on the formulation and manufacturing process. The release rate of a given product from different manufacturers is likely to be different.

Drug Product Quality and Performance Tests

A USP drug product monograph contains tests, analytical procedures, and acceptance criteria. Drug product tests are divided into two categories: (1) those that assess general quality attributes, and (2) those that assess product performance, e.g., in vitro release of the drug substance from the drug product. Quality tests assess the integrity of the dosage form, but performance tests, such as drug release, assess attributes that relate to in vivo drug performance. Taken together, quality and performance tests are intended to ensure the identity, strength, quality, purity, comparability, and performance of semisolid drug products.

Details of drug product quality tests for semisolid drug products can be found in chapter ⟨3⟩. Product performance tests for semisolid drug products are conducted to assess drug release from manufactured pharmaceutical dosage forms. In vitro performance tests for semisolid products do not, however, directly predict the in vivo performance of drugs, as the primary factor that impacts bioavailability and clinical performance are the barrier properties of the epithelia to which the product is applied (epidermal or mucosal tissues). Although product performance tests do not directly measure bioavailability and relative bioavailability (bioequivalence), they can detect in vitro changes that may correspond to altered in vivo performance of the dosage form. These changes may arise from changes in physicochemical characteristics of the drug substance and/or excipients

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or to the formulation itself, changes in the manufacturing process, shipping and storage effects, aging effects, and other formulation and/or process factors.

At present, a product performance test is available to evaluate in vitro drug release for creams, ointments, lotions, and gels. Several available apparatus can be used for this evaluation, including the vertical diffusion cell, immersion cell, and a special cell used with USP *Apparatus 4*. Because of the significant impact of in vitro test parameters, such as release media, porous membrane and dosing, and the interaction of these parameters with a given drug product, the primary use of in vitro drug release testing is comparison testing in which any difference in delivery rate is undesirable. Drug release testing is most suitable for evaluation of small formulation and process changes, manufacturing site changes, and stability testing. The evaluation or comparison of large formulation changes may provide unmeaningful results, unless extensive validation is performed to select test parameters that ensure that the sensitivity of the test is meaningfully correlated with in vivo performance. The only required regulatory use of the in vitro release test is to determine the acceptability of minor process and/or formulation changes in approved semisolid dosage forms (see FDA *Guidance for Industry—Nonsterile Semisolid Dosage Forms—Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Release Testing and In Vivo Bioequivalence Documentation*; available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070930.pdf).

This chapter provides general information for testing in vitro performance of semisolid drug products.

IN VITRO PERFORMANCE TESTS

Theory

The diffusion cell is a reliable and reproducible means of measuring drug release from semisolid dosage forms. A thick layer of the semisolid product under evaluation is placed in contact with a medium in a reservoir, and the latter acts as a receptor when the drug substance diffuses through the formulation, across the membrane, and into the reservoir. Diffusion occurs across an inert, highly permeable support membrane. The membrane is intended to keep the product and the receptor medium separate and distinct. Membranes should offer the least possible diffusional resistance and should not be rate controlling. Samples are withdrawn from the receptor chamber, typically at 1-h intervals over a 4–6 h period.

After a short lag period, release of drug from the semisolid dosage form is kinetically described by diffusion of a chemical out of a semi-infinite medium into a sink. The momentary release rate tracks the depth of penetration of the forming gradient within the semisolid. Beginning at the moment when the receding boundary layer's diffusional resistance assumes dominance of the kinetics of release, the amount of the drug released, m, becomes proportional to $\sqrt{t}$ (where t = time) for solution, suspension, or emulsion semisolid system alike. The momentary rate of drug release, dm/dt, becomes proportional to $1/\sqrt{t}$, which reflects the slowing of drug release with the passage of time. The reservoir is kept large so that over the entire course of the experiment, the concentration of the drug released into a medium remains highly dilute relative to the concentration of drug dissolved in the semisolid. In these circumstances, drug release is said to take place into a diffusional sink.

When a drug is totally in solution in the dosage form, the amount of drug released as a function of time can be described by Equation 1:

$$m = 2 \times C_0 \sqrt{\frac{Dt}{\pi}}$$

where m is the amount of drug released into the sink per cm², C_0 is the drug concentration in the releasing matrix, and D is the drug diffusion coefficient through the matrix.

A plot of m versus $\sqrt{t}$ will be linear with a slope of:

$$2 \times C_0 \sqrt{\frac{D}{\pi}}$$

Equation 2 describes drug release when the drug is in the form of a suspension in the dosage form:

$$m = \sqrt{2 \times D_m \times C_s \left(Q - \frac{C_s}{2}\right) \times t}$$

where D_m is the drug diffusion coefficient in the semisolid matrix, C_s is the drug solubility in the releasing matrix, and Q is the total amount of the drug in solution and suspended in the matrix. When $Q >> C_s$, Equation 2 simplifies to Equation 3:

$$m = \sqrt{2 \times Q \times D_m \times C_s \times t}$$

A plot of m versus $\sqrt{t}$ will be linear with a slope of:

$$m = \sqrt{2 \times Q \times D_m \times C_s}$$

Coarse particles may dissolve so slowly that the moving boundary layer recedes to some extent behind the particles. That situation introduces noticeable curvature in the $\sqrt{t}$ plot because of a particle size effect.

During release rate experiments, reasonable attempts should be made to keep the composition of the formulation intact over the releasing period.

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Drug Release Rate Determination Using Vertical Diffusion Cell Apparatus

Many vertical diffusion cell (VDC) systems are composed of 6-cell units. Each VDC cell assembly consists of two chambers (a donor chamber and a receptor chamber) separated by a membrane and held together by a clamp, screw top, or other means (see *Figure 1–Model A*, *Figure 2–Model B*, and *Figure 3–Model C*). Other diffusion cells that are similar in general design also can be used. In the donor chamber, the semisolid dosage form sample sits on a synthetic, inert, highly permeable support membrane. For the VDC Model A, the sample sits on the support membrane within the cavity of the sample chamber covered with a glass disk.

Typically, amounts of the semisolid sample NLT 200 mg are used. Diffusive communication between the semisolid sample and the reservoir takes place through the support membrane. The membrane is intended to keep the drug product sample and receptor medium separate and distinct. A heating jacket or a suitable device should be used to maintain the temperature within the cell. The release rate experiment is carried out at 32 ± 1°, except in the case of vaginal drug products for which the temperature should be 37 ± 1°. Usually a set of 6 cell assemblies are operated together at one time (i.e., single run). Sampling generally is performed over a 4–6 h time period, and the volume withdrawn is replaced with stock receptor medium. To achieve sink condition, the receptor medium must have a high capacity to dissolve the drug, and the drug concentration in the receptor medium at the end of the test ideally should be as low as possible. For each cell, the amount of drug released (μg/cm²) at each sampling time (t_1, t_2, etc.) is determined, and the cumulative amount released plotted versus $\sqrt{t}$. The slope of the resulting line is a measure of the rate of drug release. The test is often conducted with a group of 6 or 12 cells per test run. The average of 6 slopes for each test and reference product is a measure of the drug release rate from the dosage form.

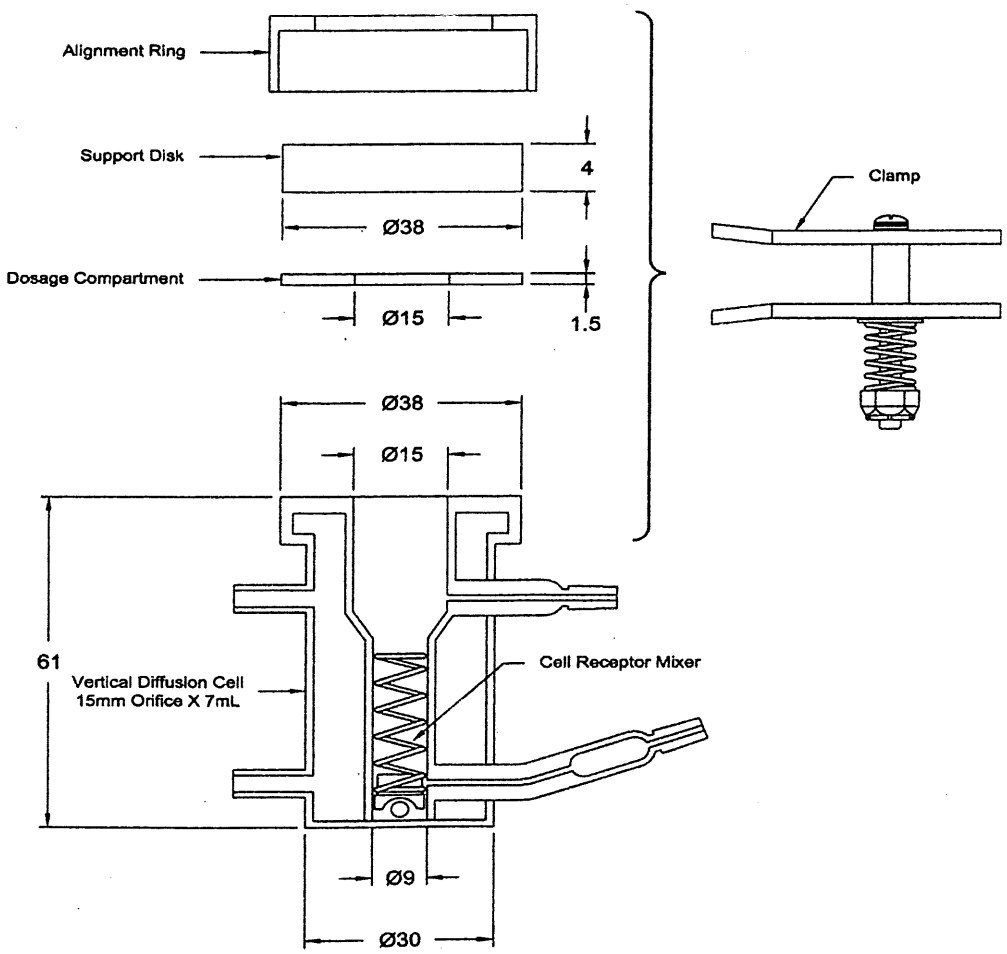

Figure 1. Vertical diffusion cell–Model A (All dimensions are in mm. All diameters are ±0.5 mm. All lengths are ±2 mm).

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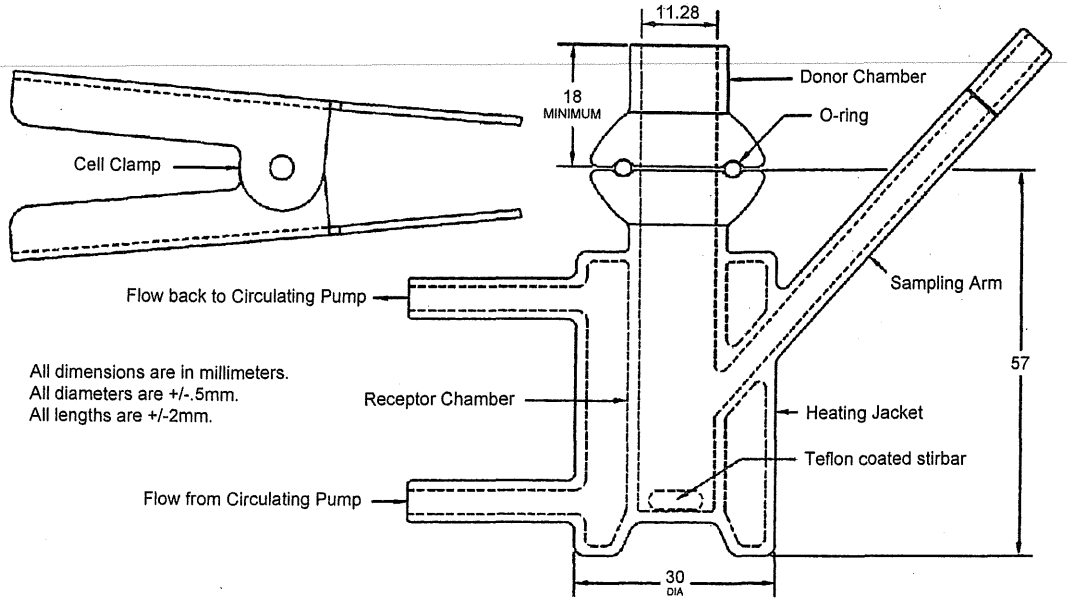

Figure 2. Vertical diffusion cell–Model B (All dimensions are in mm. All diameters are ±0.5 mm. All lengths are ±2 mm).

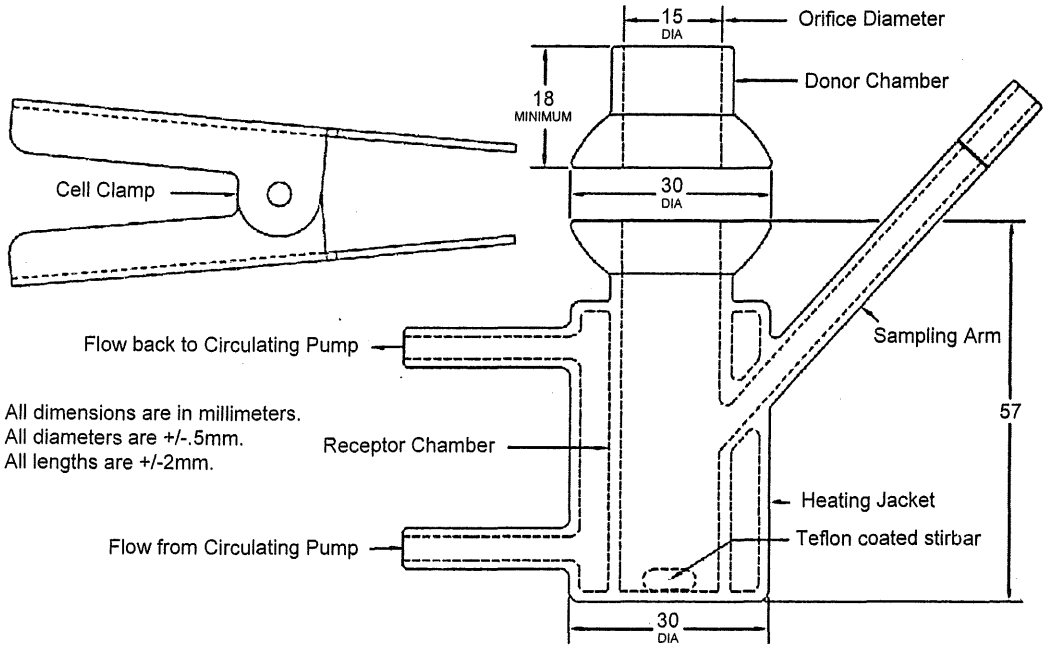

Figure 3. Vertical diffusion cell–Model C (All dimensions are in mm. All diameters are ±0.5 mm. All lengths are ±2 mm).

The VDC body (i.e., donor and receptor chambers) usually is made from borosilicate glass, although different materials may be used to manufacture the body and other parts of the VDC assembly. It is recommended that the cell assembly materials should not significantly react with, adsorb to, or absorb the test product or samples. The semisolid dosage form is placed on a membrane within the cavity of the dosage chamber that can be occluded. The diameters of the orifices of the donor chamber and receptor chamber, which define the dosage delivery surface area for the test, should be sized within ±5% of the specified diameter. The diameter of the donor and receptor chamber orifices may vary depending on the application. The receptor chamber orifice should never be smaller than the orifice of the donor chamber but should be fabricated to the same size as the donor chamber orifice. The design of the VDC should facilitate proper alignment of the donor chamber and the receptor orifice. The receptor chamber should be manufactured consistently with uniform height and geometry. All the cells should have the same nominal value, and the true volume should be measured for each individual cell. Care should be taken to minimize the intercell volume variability.

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For the test, the VDC units are typically positioned in a stirrer rack (not depicted) that holds multiple VDC units (e.g., in sets of 6) in the correct orientation, providing magnetic stirring at a calibrated rate and facilitating the supply of circulating heated water flow to the water jacket of the VDC. The VDC rack is typically connected to a thermostatically controlled water bath recirculator.

The water from the circulating pump flows into the VDC heating jacket from the lower port and flows out from the upper port to facilitate the removal of any air bubbles formed in the heating jacket. A magnetic nonstick (Teflon-coated) stirring bar in the receptor chamber is used as the internal stirring mechanism. Aliquots of the receptor medium are drawn via the sampling arm at intervals throughout the test, and an equivalent volume of stock receptor medium replaced to the level of the calibration mark on the sampling arm.

MODEL A

The thickness of the sample chamber normally is 1.5 mm. This thickness should be sized within ±10% of the specified thickness. The glass support disk is used to occlude the semisolid dosage form. A receptor cell mixer and stirrer magnet are used as the internal stirring mechanism.

MODELS B AND C

Classic styles of VDC are depicted in *Figure 2* and *Figure 3* and illustrate minor design variations among qualified models.

Test Procedures: General

Before initiating testing, analysts should determine the volume of each VDC with the internal stirring device in place. During the entire test, the temperature of the receptor medium should be maintained at 32 ± 1°, or 37 ± 1° for vaginal preparations. The rotational stirring rate tolerance should be ±10% of the rate in the method (normally 600 rpm). The rate of stirring should ensure adequate mixing of the receptor medium during the test period. Samples from each cell should be obtained at the specified times in the method within a tolerance of ±2 min. Unless the method specifies otherwise, the qualification of the apparatus has been verified when analysts determine that the test temperature and stirring rate are within their specified requirements and a satisfactory performance verification test (i.e., drug release rate) results. Unless otherwise specified in the method, degas the medium using an appropriate technique. Determine the amount of drug in the receptor medium sample aliquots using a validated analytical procedure.

The following sections provide instructions for proper use of Models A, B, and C.

TEST PROCEDURES: MODEL A

With the stirring mechanism in place, fill the receptor chamber with the specified medium with the stirrers rotating and a positive meniscus covering the top of each cell. Allow time for the medium to equilibrate to the specified temperature. Stop the stirrer before placing the test sample on the cell. If necessary, saturate the membrane in the specified medium (generally the receptor medium) for 30 min. Place the membrane on the donor chamber, and invert. Apply the material to be tested into the cavity of the sample chamber, spreading the semisolid out to fill the entire cavity of the sample chamber. Place the filled sample chamber on the receptor chamber with the membrane down and in contact with the receptor medium. During this procedure it is important to ensure that there are no bubbles beneath the membrane. Then assemble the complete cell. When the assembly of all donor and receptor chambers and remaining cell components (i.e., disk, alignment ring, and clamp) have been completed, turn on the stirring device, which constitutes the start of the test or time zero. Sampling is generally performed over a 4–6 h time period. Follow the specified sampling procedure, and collect an aliquot from each cell receptor chamber for analysis. With the stirrer stopped and using a syringe, replace the withdrawn volume with stock receptor medium warmed to the specific temperature, and resume stirring. During the sampling and medium replenishment process(es), ensure that bubbles are not introduced into the cell.

TEST PROCEDURES: MODELS B AND C

A nonstick (Teflon-coated) stir bar is placed within the receptor chamber of the VDC. The membrane specified in the test method is clamped atop the O-ring, if present, between the aligned donor and receptor chambers of the VDC. The exposed periphery of the joint between the donor and receptor compartments is sealed (e.g., circumscribed by stretched paraffin wax film).

The receptor chamber is filled with receptor medium via the sampling arm, unless it is already filled before the membrane is mounted. The VDC assembly is tilted in multiple orientations and inspected to ensure that any air bubbles trapped beneath the membrane, or within the receptor chamber, can escape via the sampling arm port. The volume of receptor medium is adjusted to the calibrated level marked on the sampling arm port. The membrane is allowed to equilibrate with the receptor medium, in situ, for at least 30 min prior to the application of the dosage form, or may be pre-incubated with a wetting solution (typically the receptor medium), as specified in the test method.

The VDC units are positioned in a stirrer rack. It is recommended that about 10–20 cm of slack should be available in the tubing connecting the ports of the VDC water jacket to the VDC rack to facilitate subsequent manipulation of the VDC during the test. The temperature set point of the water bath is adjusted before dosing so that the membrane is at the correct temperature. This can be verified by measuring the membrane temperature before dosing, using a calibrated infrared thermometer.

The stirring is initiated and can be maintained continuously throughout the test. The dosage form is evenly dispensed directly onto the membrane surface. The amount of sample recommended is NLT approximately 1.0 mL/cm^2 or 1.0 g/cm^2 to ensure a pseudo-infinite dose condition. Spreading of the sample typically starts at the outer edge and proceeds in an inward spiral

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pattern to assure full coverage of the edges of the dose area without air gaps. The placement of the sample constitutes the start of the test or time zero. The donor chamber is subsequently sealed with an occlusive film to prevent loss of any volatile components of the test formulation. The underside of the membrane is checked for air bubbles and, if any are seen, they are eliminated by tilting the apparatus in a manner that allows the air bubbles to escape. The receptor volume is confirmed at the calibrated volume mark and adjusted as necessary.

Before sample collections, typically every hour over the 4–6 h period following the introduction of the sample, the volume in the sampling arm is confirmed approximately 10 min before sampling and is adjusted to the calibration mark on the sampling arm as necessary. At predetermined intervals after starting the test, typically hourly for 6 h, analysts collect aliquots of the receptor medium (e.g., 150 µL) via the sampling arm, drawing from the well-mixed center of the receptor chamber. The VDC assembly is inspected for air bubbles, which are eliminated as necessary. Receptor medium is replaced to bring the receptor volume back to the level indicated on the sampling arm of the VDC.

Drug Release Rate Determination Using Immersion Cell Apparatus

The cell consists of the following components (see *Figure 4* and *Figure 5* for Model A, and *Figure 6* and *Figure 7* for Model B): a retaining or lock ring that secures the membrane to the cell body and ensures full contact with the sample; a washer that provides a leakproof seal between membrane, retaining ring, and cell body; the membrane (usually a synthetic membrane) that should retain the sample in the sample compartment; and the cell body that provides a variable depth reservoir for the sample. Model A also has an adjustment plate that allows operators to vary the volume of the reservoir within the cell body. The plate can be placed at the appropriate height for each test and can be completely removed to facilitate cleaning. An O-ring paired with the adjustment plate prevents leakage.

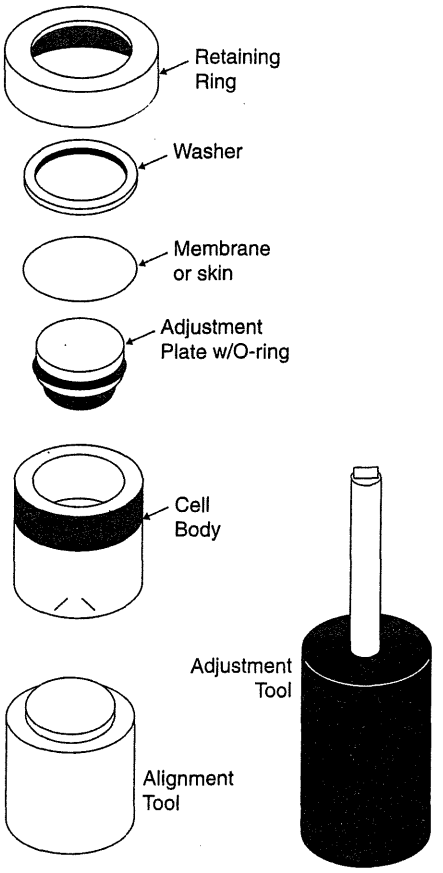

Figure 4. Immersion cell–Model A–Cell components.

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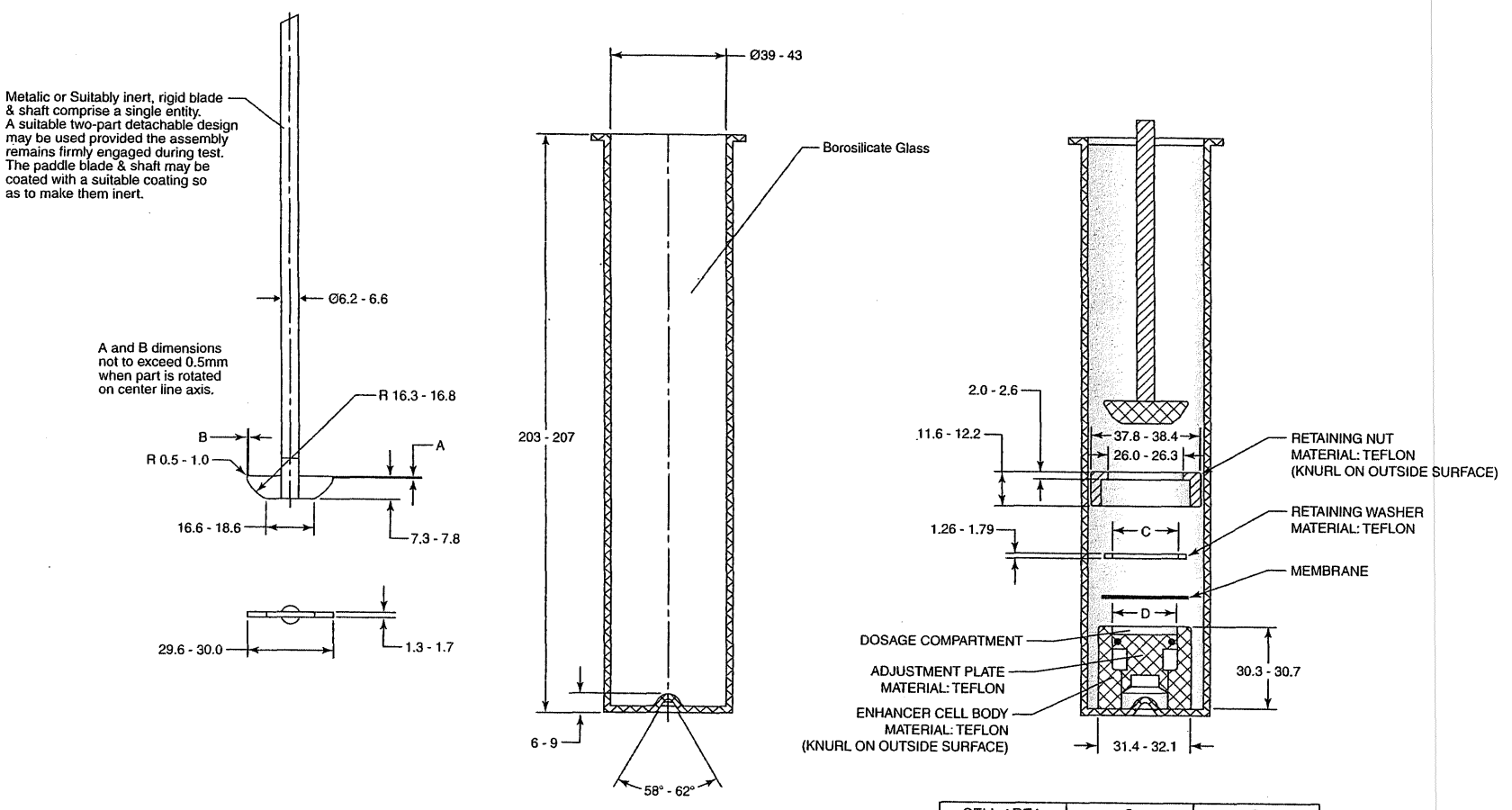
ALL UNITS ARE MILLIMETERS (mm), UNLESS OTHERWISE NOTED



CELL AREA	C	D
4 SQ CM	22.48 - 22.73	22.22 - 22.30
2 SQ CM	15.82 - 16.08	15.88 - 15.90
0.5 SQ CM	7.90 - 8.16	7.95 - 7.98

Figure 5. Immersion cell–Model A assembled in a mini vessel.

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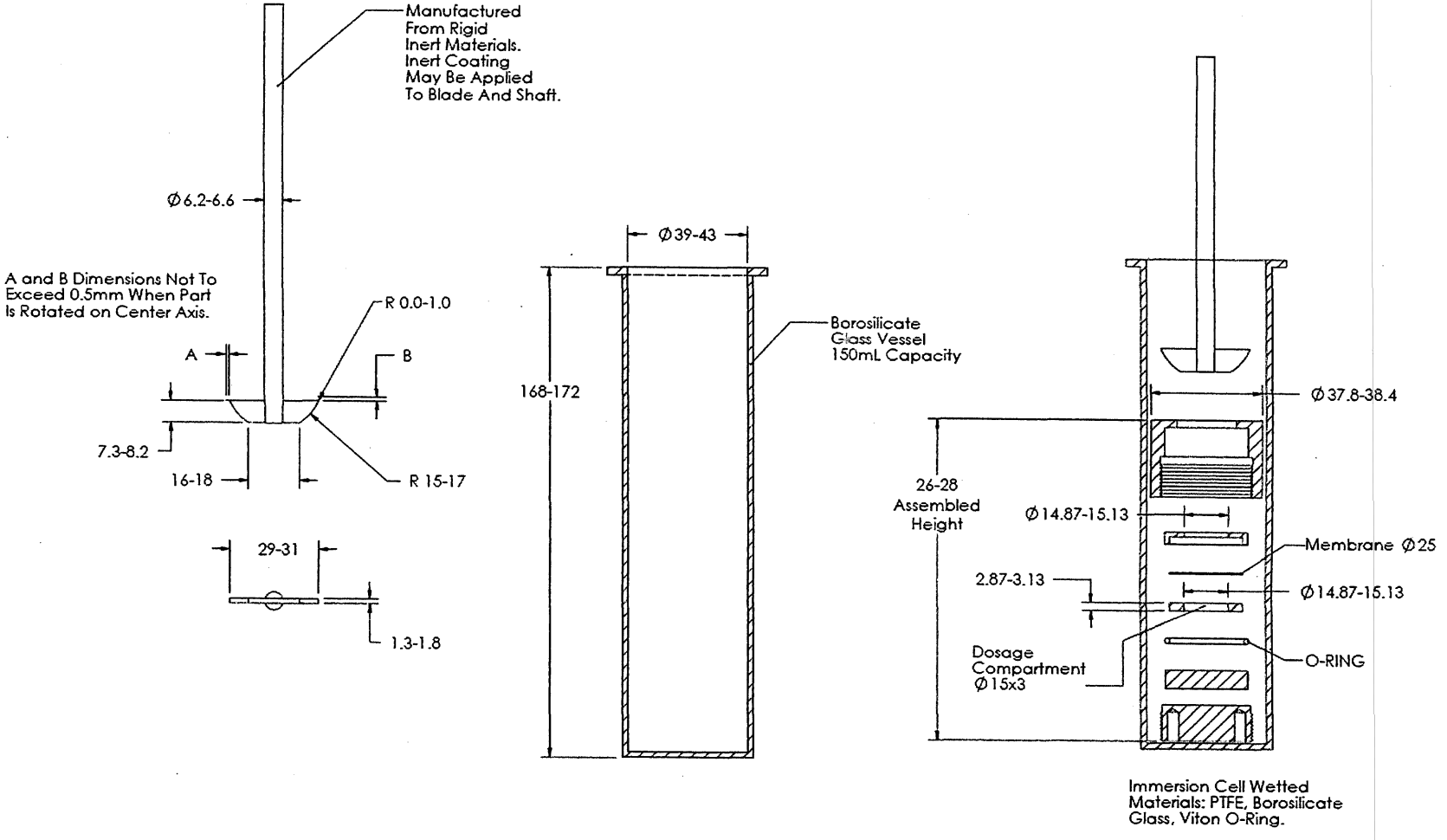

Figure 6. Immersion cell–Model B–Cell components.

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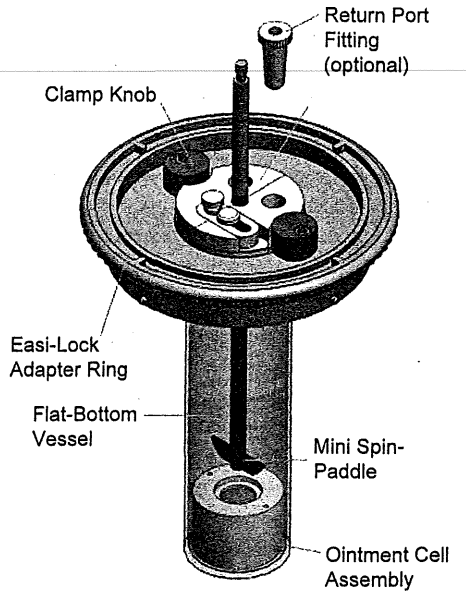

Figure 7. Immersion cell–Model B assembled in a vessel.

The immersion cell can be used with USP *Apparatus 2* (see general chapter *Dissolution* ⟨711⟩) with vessel volumes that vary from 100 mL up to 4 L, but the 150- or 200-mL vessels are the most commonly used. A flat-bottom variation of the 150- or 200-mL vessel can be used to avoid the issue of dead space under the cell when it is used in a round-bottom vessel. If analysts are going to use a 150- or 200-mL vessel with USP *Apparatus 2*, then the appropriate modifications must be made, including holders for the small-volume vessels and replacement of the standard paddle with the appropriate paddle. It also may require repositioning of any automated sampling device and/or manifold. The water bath or vessel heater should be set to have the medium temperature at 32.0 ± 0.5° or 37.0 ± 0.5°.

Before loading the cells and placing the medium in the vessel, set the paddle height, which is 1.0 ± 0.2 cm above the surface of the membrane. All other operational parameters, such as level, vibration, wobble, etc., should be set at the same conditions defined for USP *Apparatus 2*. The small-volume condition is qualified by first using the standard *Apparatus 2* setup and *Performance Verification Test, Apparatus 1 and 2* (see *Dissolution* ⟨711⟩).

Cut the membrane to an appropriate size. If necessary, soak the membrane in the receptor medium for at least 30 min before loading. If the membrane is thick, a longer soaking time period may be necessary. Prepare the immersion cell components as specified by the device manufacturer.

Fill the reservoir dosage area with the sample under test. Ensure that the reservoir is filled to the top in order to minimize the possibility of air bubble formation between the surface of the sample and the membrane. A uniform surface can be obtained with the aid of a spatula. The typical quantity of sample is between 300 mg and 2 g, depending on the type of immersion cell used. An excess of sample is needed to obtain a steady-state drug release rate. Using forceps or tweezers, remove the membrane from the soaking medium and place it over the top of the sample compartment. Ensure that the membrane is free of wrinkles. Assemble the immersion cell components as specified by the device manufacturer. Carefully place the completed assembly into the bottom of the dissolution vessel with the membrane facing up. The appropriate preheated medium may be preloaded in the vessel or can be added after immersion of the immersion cell to start the test. Samples from at least 5 time points should be obtained in the steady-state (linear) portion of the drug release profile. The data points are cumulative and expressed as concentration per surface area, typically per cm^2, as a function of the square root of time. Sampling is generally performed over a 4–6 h time period. The slope of the line is the in vitro release rate of drug from the product. At the end of the test period, dismantle the cell and examine the contents for anything unusual that could explain any anomalous data (e.g., leaks, bubbles, etc.).

QUALIFICATION

USP *Apparatus 2* should be qualified according to the procedure described in *Dissolution* ⟨711⟩.

Drug Release Determination Using USP Apparatus 4 (Flow-Through Cell)

The adapter for semisolid dosage forms (see *Figure 8*) is used with the 22.6-mm cell of USP *Apparatus 4* described in *Dissolution* ⟨711⟩. The adapter consists of a reservoir and a ring to hold the membrane. The reservoir is available in different sizes that can accommodate from 400 to 1200 µL of product. The use of the USP *Apparatus 4* cells ensures control of temperature and hydrodynamics. The temperature can be maintained either at 32.0 ± 0.5° or 37.0 ± 0.5°, depending on the intended site of the administration of the formulation. The flow rate should comply with the requirements of *Dissolution* ⟨711⟩ with a sinusoidal flow profile with a pulsation of 120 ± 10 pulses/min and a precision of ±5% of the nominal flow rate.

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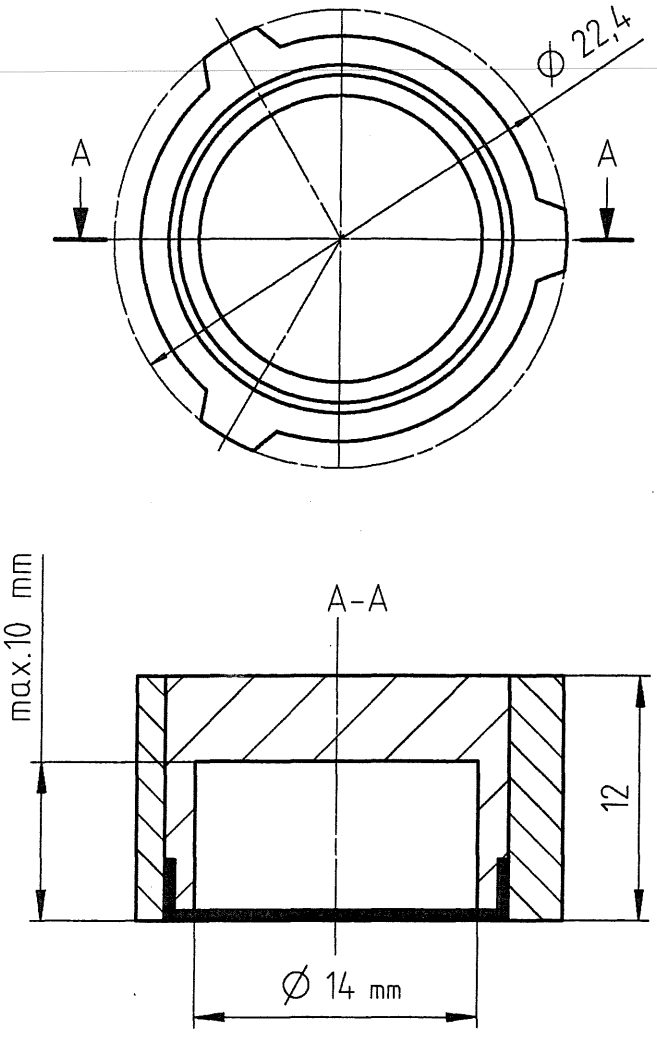

Figure 8. Adapter for topical dosage forms in USP *Apparatus 4* (All dimensions are in mm).

PROCEDURE

The membrane, which may be soaked in the receptor medium beforehand, is loaded in the membrane ring using the provided tool. The membrane should be large enough to overlap the top edge of the reservoir body with a diameter of 18 mm. The sample is loaded into the reservoir. The other side of the tool can be used to hold the reservoir while loading the sample. If necessary, the excess of sample can be removed using a spatula. Screw the membrane ring onto the sample reservoir. Ensure that the membrane is free of wrinkles while screwing.

Remove the semisolid sample adapter from the tool, and slide it into the cylindrical part of the 22.6-mm cell with the membrane facing downward. Vertical positioning within the cell can be adjusted using the tablet holder scoring, if desired (see *Figure 9*). If the lower position is chosen, release can be higher due to the proximity to the flow inlet. The system is typically configured as a closed system (see *Figure 10*), but in some cases, an open system can be used. The prepared cell is inserted in a heating jacket.

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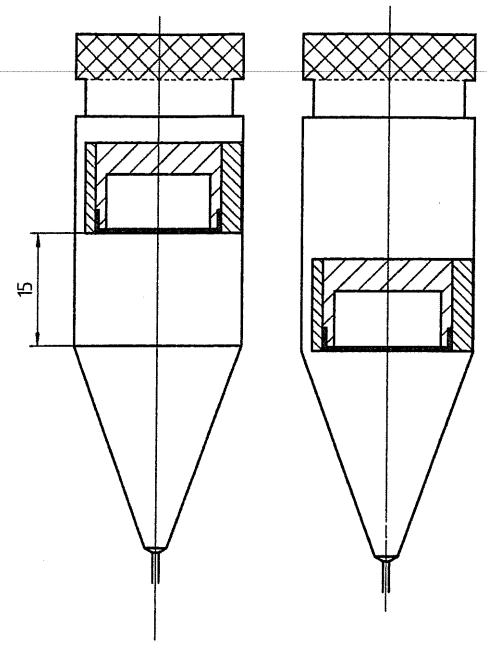

Figure 9. Vertical positioning of the insert using the tablet holder scoring (all dimensions are in mm).

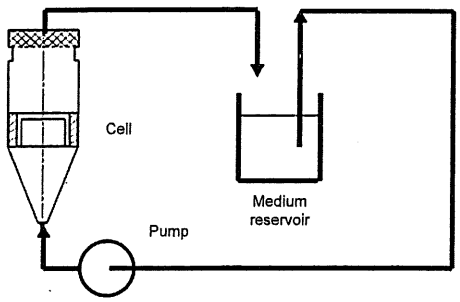

Figure 10. Closed system configuration.

The defined volume of release medium is introduced in the reservoir. Unless otherwise specified, the medium should be deaerated in order to minimize the risk of air bubbles. A deaeration procedure is described in *Dissolution* ⟨711⟩, but other validated deaeration techniques can be used. The reservoir can be adapted to the volume needed in order to achieve sink conditions and to ensure precision of the analytical method. Typical volumes range from 50 to 1000 mL, but values above and below this range also can be used as the formulation demands.

When the pump is switched on, the medium will be pumped through the cell. This represents the time zero of the test. Typical flow rates are 16 mL/min and 24 mL/min, but flow rate is a method-development parameter and must be optimized accordingly. The flow passing through the cell ensures both agitation and renewal of the receptor medium at the interface with the membrane.

Sampling can be performed either manually or automatically directly from the medium reservoir, thus ensuring no interference with the flow cell and its contents. An automated fraction collector may be appropriate for release periods longer than 6 h. After quantification, plot the amount of drug release per surface area versus the square root of time, with the slope of the line representing the in vitro release rate.

Calculation of Rate and Amount of Drug Released

Calculate the drug release rate using the following steps.

Amount released (µg/cm²) at a given time (t_1, t_2, etc.) (AR_i) is calculated for each sample:

$$\text{Amount released at } t_1 AR_1 = (A_{U1}/A_S) \times C_S \times 1000 \times (V_C/A_0)$$

$$\text{Amount released at } t_2 AR_2 = (A_{U2}/A_S) \times C_S \times 1000 \times (V_C/A_0) + [AR_1 \times (V_S/V_C)]$$

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$$AR_n = (A_{Un}/A_S) \times C_S \times 1000 \times (V_C/A_O) + \left[(V_S/V_C) \times \sum_{i=1}^{n-1} \left(\frac{A_{U(n-1)}}{A_S} \right) \times C_S \times 1000 \times V_C/A_O \right]$$

AR = amount of drug released (µg/cm²)
A_U = response (e.g., peak area, or peak height or absorbance) from the *Sample solution*
A_S = average response (e.g., peak area, or peak height or absorbance) from the *Standard solution*
C_S = concentration of the *Standard solution* (mg/mL)
V_C = volume of the diffusion cell (mL)
A_O = area of the orifice (cm²)
V_S = volume of sample taken (mL)

For each cell, the individual amount of drug released is plotted versus the square root of time. The slope of the resulting line is the rate of drug release. The average of 6 slopes for each test and reference products represents the drug release rate of the dosage form, and serves as the standard for the drug product.

Application of Drug Release

The product performance test can be used to assess sameness of the drug product after post-approval changes. Because common testing artifacts, such as air bubbles and membrane defects, yield measurements that are not normally distributed, a nonparametric statistical technique is used to evaluate the test results. The Mann-Whitney U test is used to calculate the 90% confidence interval for the ratio of the slopes between the test and the reference batches. This is illustrated by the following example in which the initial drug product batch is referred to as the reference batch (*R*) and the changed or subsequent batch is referred to as the test batch (*T*). The individual amounts of drug released from *R* are plotted versus the square root of time, and the resulting slopes are determined. Those are the reference slopes. The process is repeated for the test batch (*T*).

The *T*/*R* slope ratios are calculated for each test-to-reference slope. This procedure is facilitated with a table where the values for the slopes for test and reference batches are listed down the left side and across the top of the table, respectively. The *T*/*R* slope ratios are then determined. See *Table 1*.

Table 1. Comparison of Test and Reference Slopes

	RS1	RS2	RS3	RS4	RS5	RS6
TS1	TS1/RS1	TS1/RS2	TS1/RS3	TS1/RS4	TS1/RS5	TS1/RS6
TS2	TS2/RS1	TS2/RS2	TS2/RS3	TS2/RS4	TS2/RS5	TS2/RS6
TS3	TS3/RS1	TS3/RS2	TS3/RS3	TS3/RS4	TS3/RS5	TS3/RS6
TS4	TS4/RS1	TS4/RS2	TS4/RS3	TS4/RS4	TS4/RS5	TS4/RS6
TS5	TS5/RS1	TS5/RS2	TS5/RS3	TS5/RS4	TS5/RS5	TS5/RS6
TS6	TS6/RS1	TS6/RS2	TS6/RS3	TS6/RS4	TS6/RS5	TS6/RS6

After the *T*/*R* ratios have been calculated, they are ordered from the lowest to the highest. The 8th and 29th *T*/*R* ratios are identified and converted to percent (multiplied by 100). These values represent the 90% confidence interval for the ratio of test to reference release rates. To pass first stage testing, those ratios must be within the range of 75%–133.33%.

If the results do not meet this criterion, four additional tests of 6 cells should be performed, resulting in 12 additional slope determinations for each product tested. The *T*/*R* slope ratios for all 18 slopes for each product tested are determined. All 324 individual *T*/*R* slope ratios are ordered from the lowest to the highest. To pass this second stage testing, the 110th and 215th slope ratios, representing the 90% confidence interval, must be within the range of 75%–133.33%.

⟨1730⟩ PLASMA SPECTROCHEMISTRY—THEORY AND PRACTICE

OUTLINE

1. *Introduction*
2. *Sample Preparation*
3. *Sample Introduction*
4. *Standard Preparation*
5. *Inductively Coupled Plasma (ICP)*
6. *Inductively Coupled Plasma Optical Emission Spectroscopy (ICP–OES)*
   - *6.1 Calibration*
   - *6.2 Standardization*
   - *6.3 Procedure*

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7. *Inductively Coupled Plasma Mass Spectrometry (ICP–MS)*
7.1 Calibration
7.2 Standardization
7.3 Procedure
8. *Glossary*
9. *References*

1. INTRODUCTION

The purpose of this general chapter is to provide a general overview of fundamental principles, instrumentation and application of inductively coupled plasma optical emission spectroscopy (ICP–OES) and inductively coupled plasma mass spectrometry (ICP–MS). It is the companion chapter to *Plasma Spectrochemistry* ⟨730⟩. A glossary of terms is located at the end of this general chapter.

2. SAMPLE PREPARATION

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP–OES or ICP–MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP–OES and ICP–MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. These samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid can be used, but great care must be taken when using this acid to ensure the safety of the analyst, and to protect the components of the sample introduction system, specifically: peristaltic pump tubing, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric acid-tolerant materials. Proper safety procedures must be followed to protect the analyst, as well. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to: the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents, or any solvent that is compatible with the instrumentation.

When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP–OES and ICP–MS analyses in cases where accuracy and precision are not adequate. The use of an internal standard should be considered the rule, rather than the exception, in the case of ICP–MS analyses. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can be employed. These include hot-plate digestion and microwave-assisted digestions, including open-vessel and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP–MS is employed. Deionized water must be at least 18 MΩ. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are free of metals, use organic solvents of the highest quality possible with regard to metal contaminants. Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open-vessel or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation. Additionally, it may be necessary to extract the analyte(s) of interest, should a sample not completely dissolve. In such an instance, the validity of the extract must be demonstrated by means of spike and recovery studies.

It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical lab ware used in ICP–OES and ICP–MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the absorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can lead to inaccurate results.

The use of lab ware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

3. SAMPLE INTRODUCTION

There are two ways to introduce the sample into the nebulizer: by means of a peristaltic pump, and by self-aspiration. The peristaltic pump is preferred and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same irrespective of sample viscosity. The speed setting of the peristaltic pump should remain constant throughout an analysis during the time period when readings are being taken by the instrument. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

The purpose of a nebulizer is to generate very small droplets of the sample, with the goal of generating a fine aerosol mist. A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers.

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Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli, or Venturi, effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1%–2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP.

As with nebulizers, there is more than one type of spray chamber available for use with ICP–OES or ICP–MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered. Gas and liquid chromatography systems can be interfaced with ICP–OES and ICP–MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass spectrometry.

Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision for the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA–ICP–OES and LA–ICP–MS are better suited for qualitative analyses of pharmaceutical compounds because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated, through appropriate method validation, that the available standards are adequate (*1*).

4. STANDARD PREPARATION

Single- or multi-element standard solutions, whose concentrations are traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials, as appropriate, and their concentrations can be determined independently. Working standard solutions, especially those used for ultra-trace analyses, may have limited shelf life, depending on the analyte in question, the type of storage container, the solution's concentration, and the storage conditions. As a general rule, working standard solutions with concentrations less than 10 ppm (w/v) should be retained for NMT 24 h unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards, blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions should be used for ICP–OES or ICP–MS. The method of standard additions may be necessary, even with the use of matrix-matched solutions and internal standards. In any event, the selection of an appropriate internal standard should consider the analytes in question, their ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the *x*-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

Optimization of the ICP–OES or ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. Selection of wavelengths or isotopes must be carefully considered. The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP–OES and ICP–MS selenium and arsenic quantification measurements, which can be remedied by using the method of standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity standards.

5. INDUCTIVELY COUPLED PLASMA (ICP)

The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF) induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in the ICP. The plasma torch consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred to as the auxiliary) gas. The intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch from melting.

An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field, which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching unit serves to

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efficiently couple the RF energy from the generator to the load coil. The unit can be of either the active or the passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF generator, the energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of 6,000–10,000 K, so most covalent bonds and analyte-to-analyte interactions are eliminated.

6. INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROSCOPY (ICP–OES)

The ICP can use either an optical or a mass spectral detection system. In the former case, ICP–OES, analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a wide variety of ICP–OES systems are available, each with different capabilities as well as different advantages and disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby shortening analysis time and improving background detection and correction. Sequential systems move from one wavelength to the next (sometimes referred to as slewing) to perform analyses, and often provide a larger number of analytical lines from which to choose.

Modern instruments typically use array detectors as detection devices. Array detectors, including charge-coupled devices and charge-injection devices, have detectors assembled on a chip, making it possible to combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most powerful spectrometers, providing rapid analysis and a wide selection of analytical lines. Some instruments may use photomultiplier tubes (PMT's) for detection. PMT's are best-suited for simultaneous analyses; however, the use of PMT's is quickly waning, and array detectors are more commonly found.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision); however, it also incurs greater matrix and spectral interferences. Axial plasmas normally require the use of a shear gas, which effectively removes the coldest part of the plasma to help reduce self-absorption. Methods validated on an instrument with a radial configuration may not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, the analyte in question, the analytical wavelength(s) used, the cost of instrumentation, the required sensitivity, and the type of instrumentation available in a given laboratory.

Regardless of torch configuration or detector technology, ICP–OES is a technique that provides a qualitative and/or quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for the background emission from the plasma. Sample concentration measurements are usually determined from a working curve of known standards over the concentration range of interest. It is, however, possible to perform a single-point calibration under certain circumstances, such as limit tests, if the method has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in the ICP are rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because "wings" of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP–OES.

The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap. Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at a baseline level away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a background correction can also be performed with certain types of ICP–OES spectrometers. One simple way to avoid spectral interferences is to select an analytical line that is free of interferences, if possible.

The selection of the analytical spectral line is critical to the success of the ICP–OES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used or the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument and select alternative wavelengths based on manufacturer recommendations or published wavelength tables (*2, 3, 4, 5, 6*). Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

The analysis of the Group I elements can pose some challenges. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the fractional ionization that can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer

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gas flow. It could be necessary to reduce the peristaltic pump speed, and alter the selection of the spray chamber. When using organic solvents, it could be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

6.1 Calibration

The wavelength accuracy for ICP–OES detection must comply with the manufacturer's applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP–OES instrument should be followed.

6.2 Standardization

The instrument must be standardized for quantitation at time of use. Because ICP–OES is a technique generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests, as well as other analyses, on production materials and final products if the method has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of single-point standardization is also acceptable for qualitative ICP–OES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantitation.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. The plot of the analyte concentration against the known concentrations of components is usually performed automatically by the instrument.

It is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantitation, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may establish other criteria.

6.3 Procedure

It is important to follow the procedure for the instrument parameters, as directed in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Because of differences in manufacturers' equipment configurations, the manufacturer's suggested default conditions could be used and modified as needed. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions, which is often calculation directly by the instrument.

7. INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP–MS)

When using the ICP–MS, analytes are detected directly at their atomic masses. Because these masses must be charged to be detected in ICP–MS, the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–OES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Additionally, high-resolution sector field instruments and time of flight-based instruments are available, as are multiple quadrupole systems. Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample.

Ions are generated from the analyte atoms by the plasma, and ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, "skims" the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held in milliTorr ranges. Lastly, the skimmed ions pass a third-stage orifice to enter a zone held near microTorr pressures, where they encounter ion optics and are passed into the mass spectrometer. The pressure differences aid in moving the ions along and into the mass spectrometer, which separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units.

Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interferences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isobaric interferences and correction factors can be used, and are often pre-programmed into an instrument.

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ICP–MS generally offers considerably better detection limits than ICP–OES, largely because of the extremely low background noise that it generates. This ability is a major advantage of ICP–MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP–MS. As a general rule, ICP–MS as a technique requires that samples contain significantly less total dissolved solids than does ICP–OES.

The selection of the analytical mass to use is critical to the success of the ICP–MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts could choose to start with masses recommended by the manufacturer of their particular instrument and select alternate masses based on manufacturer's recommendations or published tables of naturally occurring isotopes (*7*).

Optimization of the ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it could be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.

7.1 Calibration

The mass spectral accuracy for ICP–MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP–MS instrument.

7.2 Standardization

The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP–MS is generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the method has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, which are normally performed by the instrument. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the *x*-intercept multiplied by any dilution factor is the concentration of the analyte in the sample. In many instances, the instrument will perform this calculation automatically after being programmed to use the method of standard additions.

7.3 Procedure

Follow the procedure for the detection mode and instrument parameters for ICP–MS, as directed in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers' equipment configurations, the analyst could begin with the manufacturer's suggested default conditions and modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as a single result. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. With modern instruments, this calculation is often performed by the instrument.

8. GLOSSARY

Auxiliary gas: See *Intermediate (or auxiliary) gas.*

Axial viewing: A configuration of the plasma for AES in which the plasma is directed toward the spectrometer optical path, also called "end-on viewing."

Central (or nebulizer) gas: One of three argon gas flows in an ICP torch. The central gas is used to help create a fine mist of the sample solution when solution nebulization is employed. This fine mist is then directed through the central tube of the torch and into the plasma.

Collision cell: A design feature of some ICP–MS instruments. Collision cells are used to reduce interferences from argon species or polyatomic ions and facilitate the analysis of elements that might be affected by those interferences.

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Cool plasma: Plasma conditions used for ICP–MS that result in a plasma that is cooler than that normally used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.

Coolant gas: See *Outer (or coolant or plasma) gas.*

Forward power: The number of watts used to ignite and sustain the plasma during an analysis. Forward power requirements may vary, depending on sample matrix and analyte.

Intermediate (or auxiliary) gas: Gas used to "lift" the plasma off the surface of the torch, thereby preventing melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.

Internal standard: An element added to or present in the same concentration in blanks, standards, and samples to act as an intensity reference for the analysis. An internal standard should be used for ICP–AES work and must always be used for quantitative ICP–MS analyses.

Lateral viewing: See *Radial viewing.*

***m*:** The ion mass of interest.

Multiply-charged ions: Atoms that, when subjected to the high-ionization temperature of the ICP, can form doubly or triply charged ions (X^{++}, X^{+++}, etc.). When detected by MS, the apparent mass of these ions will be ½ or ⅓ that of the atomic mass.

Nebulizer: Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently sent into the ICP.

Outer (or coolant or plasma) gas: The main gas supply for the plasma.

Plasma gas: See *Outer (or coolant or plasma) gas.*

Radial viewing: A configuration of the plasma for AES in which the plasma is viewed orthogonal to the spectrometer optic path. Also called "side-on viewing." See also *Lateral viewing.*

Reaction cell: Similar to *Collision cell,* but operating on a different principle. Designed to reduce or eliminate spectral interferences. Used in ICP–MS.

Sampling cone: A metal cone (usually nickel-, aluminum-, or platinum-tipped) with a small opening, through which ionized sample material flows after leaving the plasma in ICP–MS.

Sequential: A type of detector configuration for AES or MS in which discrete emission lines or isotopic peaks are observed by scanning or hopping across the spectral range by means of a monochromator or scanning mass spectrometer.

Simultaneous: A type of detector configuration for AES or MS in which all selected emission lines or isotopic peaks are observed at the same time by using a polychromator or simultaneous mass spectrometer, offering increased analysis speed for analyses of multi-element samples.

Skimmer cone: A metal cone through which ionized sample flows after leaving the sampling cone and before entering the high-vacuum region of an ICP–MS.

Standard additions: A method used to determine the actual analyte concentration in a sample when viscosity or matrix effects might cause erroneous results.

Torch: A series of three concentric tubes, usually manufactured from quartz, in which the ICP is formed.

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⟨1735⟩ X-RAY FLUORESCENCE SPECTROMETRY—THEORY AND PRACTICE

OUTLINE

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1. INTRODUCTION

This general chapter provides information regarding the theory and acceptable practices for the consistent analysis and interpretation of X-ray fluorescence spectroscopic data. X-ray fluorescence (XRF) spectrometry is an instrumental technique based on the measurement of characteristic X-ray photons caused by the excitation of atomic inner-shell electrons by a primary X-ray source. The XRF technique can be used for both qualitative and quantitative analysis of liquids, powders, and solid materials. Although some vendors supply radioactive isotope-based source instruments, nearly all modern instruments use an X-ray tube as the source.

2. PRINCIPLES OF X-RAY FLUORESCENCE SPECTROMETRY

The X-rays produced by an X-ray tube include characteristic lines corresponding to the anode material, and a continuum also known as Bremsstrahlung radiation. Both types of X-rays can be used to excite atoms in a specimen and thus induce X-rays. The intensity of both of these types can be adjusted by the voltage and current settings of the X-ray generator. These parameters can be adjusted to optimize the flux of X-ray photons for each element of interest. Further adjustments, such as the addition of filters in the primary beam, can be used to remove undesirable and potentially interfering tube spectral lines. Secondary targets may be used to produce an excitation X-ray beam that differs from the primary X-ray tube spectrum, with the aim of achieving optimum excitation conditions. Numerous instrument designs with different geometrical configurations, for example, secondary filters and polarization, are used to optimize X-ray detection and reduce background contributions.

Although many variations exist, XRF instrumentation can be divided into two categories: wavelength-dispersive XRF (WDXRF) and energy-dispersive XRF (EDXRF) instruments. The main distinguishing factor between these two technologies is the method used to separate the spectrum emitted by all atoms in the sample according to X-ray photon energy.

The *energy* (or the *wavelength*) of the X-ray photon is characteristic of a given electron transition in an atom, and is therefore *qualitative* in nature. The *intensity* of the emitted radiation is indicative of the number of atoms in the sample, and therefore constitutes the *quantitative* nature of the technique.

3. PHYSICS OF X-RAY EXCITATION AND EMISSION

3.1 Ionization and the Photo-Electric Effect

The emission of characteristic X-ray radiation results from an electron transition between two inner shells of an atom, after ionization. After, for example, the ejection of an electron from the K-shell, the atom is ionized and the ion is left in a high-energy state, E_I, with E_I being equal to the energy required to remove the K-electron from the shell to a situation where it is at rest (no remaining kinetic energy) at infinite distance from the nucleus. This interaction between electromagnetic radiation (a photon) and an atom resulting in an electron being excited is called the *photo-electric effect*. It is typically the largest contribution to absorption of X-rays. E_I thus corresponds to the binding energy of the electron, which is identical to the energy of the atomic

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level. The excited state has a very limited life span, and will decay rapidly by a transition of an electron from an outer shell to the vacancy in the inner shell. The atom is still ionized, with an electron vacancy in the other shell. Let the energy state after this transition be represented by E_2, corresponding to the binding energy of the electron prior to the transition. The energy difference, ΔE, is represented by

$$\Delta E = E_1 - E_2 \text{ [1]}$$

and can be released in two competing processes: the Auger effect, or the emission of a photon. For XRF, obviously the latter is of interest. The energy of the photon emitted is thus equal to ΔE. The binding energies of the inner electrons are not affected by the chemical state of the atom concerned. The atomic energy levels are unique (characteristic) to the element, thus the energy difference between two given levels is also a characteristic. Therefore, the resulting X-ray photon is called a *characteristic* photon. The energy of the characteristic photons is varying in a systematic way with the atomic number Z of the elements, a fact that can be represented using Moseley's law [*1*]:

$$E_{photon} = k(Z - s)^2 \text{ [2]}$$

where k is a constant for a given series (as defined by the shell with the initial electron vacancy) and s is the screening constant. A fairly complete tabulation of the energies of characteristic radiation can be found in the work by Bearden [*2*]. Assume that the initial vacancy was created in the K-shell. After an electron transition from, for example, the L3-shell, the atom still has an electron vacancy, but in the L3-shell, while a photon with an energy E corresponding to

$$E_{photon} = E_k - E_{L3} \text{ [3]}$$

has escaped the atom. The vacancy in the L3-shell can subsequently be filled by an electron from the M5-shell (assuming that this is a high-atomic-number element). This results in a vacancy in the M5-shell that can then decay further. At the end of this cascade, the ion is back in the neutral state.

3.2 Scattering

The most important interaction between X-rays and matter is the photo-electric effect, described above. Two other mechanisms are coherent and incoherent scatter.

3.2.1 COHERENT SCATTER

Coherent scatter is elastic scattering of electromagnetic radiation by a free, charged particle. It is also known as Thomson scattering. The electric field of the incident photon accelerates the charged particle. This particle will subsequently emit radiation with the same energy as the incident photon, but travelling in a different direction.

3.2.2 INCOHERENT SCATTER

Incoherent scatter of a photon is usually called Compton scatter. Compton scatter is inelastic in nature. Both momentum and energy are conserved in the process. After Compton scattering, the electron has acquired considerable momentum (and is excited from the atom), and a photon with a longer wavelength than the incident photon is emitted (see *Figure 1*). The conservation of energy and of momentum leads to a simple relationship between the scattering angle Φ and the wavelength difference $\Delta\lambda$ between the incident and the Compton scattered photon:

$$\Delta\lambda = 0.00243 \cdot (1 - \cos\Phi) \text{ [4]}$$

Equation [4] calculates the wavelength shift in nm. The peaks of Compton scattered radiation are typically broader than those corresponding to characteristic radiation or coherently scattered radiation. The reason is that most X-ray fluorescence spectrometers do not have a collimated incident beam. The incident angle on the specimen can thus vary significantly. This contributes to wide variation in total scattering angles Φ, leading to variation in $\Delta\lambda$.

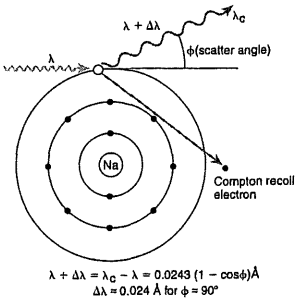

Figure 1. Compton scattering of an X-ray photon; Φ is the scattering angle (from Willis and Duncan [*3*] with permission).

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3.3 Conversion between Energy and Wavelength

Photons exhibit a wave-particle duality [4]. The energy, E, of the "particle" and the wavelength λ of the "wave" are related through Planck's constant, h:

$$E = \frac{hc}{\lambda} \quad [5]$$

where E is the photon energy; h is Planck's constant; and c is the speed of light in vacuum. By substituting these values in *Equation [5]*, and expressing photon energy E in keV and wavelength λ in nm, the following is obtained:

$$E = \frac{1.24}{\lambda} \quad [6]$$

This allows quick and easy conversion between energy and wavelength scales. Note that *shorter* wavelengths correspond to *higher* energies.

Commonly, textbooks designed around WDXRF will use wavelength units for the characteristic radiation. On the other hand, when dealing with EDXRF, the scale of choice is an energy scale with keV as the unit. Photon energy can be converted to photon wavelength and vice versa using *Equation [6]*. The *frequency* of the electromagnetic radiation (which can be calculated using c/λ or from E/h) is not used when relating to XRF analysis.

3.4 The Electromagnetic Spectrum

The X-ray range is typically the spectral range between 0.01 and 10 nm, and it spans 4 orders of magnitude. In practice, however, the range of X-rays commonly analyzed varies from about 0.04 to 1 nm.

The X-ray region of the electromagnetic spectrum can also be expressed in terms of the characteristic radiation of the elements. Most wavelength-dispersive X-ray spectrometers can be equipped to analyze characteristic lines between about 0.04 and 4.4 nm. This allows analysis of all elements in the periodic table from carbon upwards ($Z = 6$). With dedicated analyzers, the elemental range can be extended to include beryllium, although issues with limited specificity and sensitivity severely limit the practical applications for the quantitative analysis of beryllium.

3.5 Selection Rules

The transitions in the process described above are governed by quantum-mechanical selection rules. XRF radiation is observed with reasonable probability only from those transitions where

$$\Delta j = -1, 0, +1$$

and

$$\Delta l = -1 \text{ or } +1$$

where j and l are the usual quantum numbers. The correspondence between shell designations (K, L, M...) and the quantum numbers n, l, and m is given in *Table 1*. This means that in transitions such as K ← L1 and L1 ← M1, both shells with $l = 0$ for initial and final state are forbidden radiative transitions. These transitions, however, can be accompanied by the emission of an Auger electron.

Table 1. Correspondence between Shell Designations (K, L, M, N) and Quantum Numbers (*n*, *l*, *m*)

Shell	Quantum Numbers			Number of Orbitals	Subshell Designation	Number of Electrons to Fill Subshell	Total Number of Electrons in Shell
	n	*l*	*m*				
K	1	0	0	1	1s	2	2
L	2	0	0	1	2s	2	8
	2	1	−1,0,1	3	2p	6	
M	3	0	0	1	3s	2	18
	3	1	1,0,−1	3	3p	6	
	3	2	−2,−1,0,1,2	5	3d	10	
N	4	0	0	1	4s	2	32
	4	1	1,0,−1	3	4p	6	
	4	2	−2,−1,0,1,2	5	4d	10	
	4	3	−3,−2,−1,0,1,2,3	7	4f	14	

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After the initial vacancy has been created, the ion is in a highly excited state. After an electron transition, for example, K ← L3, an energy equal to the difference in the binding energies (see *Equation [3]*) between the two shells involved can be released. In the case on hand, this gives rise to the emission of a Kα photon. Photon emission is, however, only one of two competing processes. The other is the emission of an Auger electron. This is the process in which the energy is transferred to another electron, which escapes the ion with a certain kinetic energy E_{Kin}. The kinetic energy can be calculated in much the same fashion as the photon energy, using the energy levels from the shells involved. If the energy released from the K ← L3 transition is transferred to an L1 electron, the kinetic energy can be calculated from

$$E_{Kin} = E_{Auger} = E_K - E_{L1} - E_{L3} \quad [7]$$

The kinetic energy of the Auger electron is characteristic because it is made up of characteristic quantities. The vacancies created after the emission of an Auger electron can subsequently decay, either by other Auger electrons or by photons.

3.6 Fluorescence Yield

The fluorescence yield (symbol ω) is defined as the probability that a vacancy is filled through a radiative transition. For the K-shell this is simply

$$\omega_K = \frac{I_K}{n_K} \quad [8]$$

where I_K is the total number of K-shell X-ray photons and n_K is the number of vacancies created in the K-shell. For the L-shell and M-shell, a similar definition applies for each of the subshells, but the number of primary vacancies needs to be corrected for the cascade effects and the occurrence of Coster-Kronig transitions [*5*]. These are nonradiative transitions between subshells having the same principal quantum number. Data regarding values for fluorescence yields can be found in the work by Bambynek et al. [*5*] and Hubbell et al. [*6*].

3.7 Counting Statistical Error

The *counting statistical error* (CSE) is the uncertainty in the measurement of the number of photons, which is subject to a Poisson distribution. The distribution of the number of photons can be approximated by a normal distribution, because the number of photons counted is usually sufficiently large. The standard deviation σ_N of an intensity measurement of N counts is given by the square root of the number of counts:

$$\sigma_N = \sqrt{N} \quad [9]$$

Most measurements in XRF are now expressed as an intensity, I, which is simply the ratio of the number of photons counted divided by the measuring time, t. The counting statistical error on a measured intensity can be calculated using

$$\sigma_I = \sqrt{\frac{I}{t}} \quad [10]$$

This indicates that the counting statistical error on an intensity measurement becomes smaller with longer measurement times. Measuring longer on an XRF instrument (under otherwise constant conditions of measurement) reduces the relative uncertainty on the measurement. This is generally applicable until the resulting CSE becomes comparable to the *overall instrument error*. For high-end instrumentation, the order of magnitude of this overall instrument error is about 0.1% or better. Performing measurements with a CSE significantly lower than the overall instrumental error will not lead to more precise results. The CSE thus imposes a theoretical lower limit to the precision of an X-ray measurement. The contribution of the instrumental errors is added to the CSE to obtain the total error on the measurement.

The *intensity* of a photon beam is calculated from the number of photons collected divided by the time taken for the measurement, expressed in counts per second (cps) or kilocounts per second (kcps).

3.8 Detection Limit

The lower limit of detection (LLD) is defined as the concentration that will yield a positive intensity above background intensity with a given confidence level. For analysis near the detection limit, the intensity of the peak will be comparable to the intensity of the background. For example, a net signal that is 3 times larger than the CSE of the background will satisfy this criterion with 99.7% confidence. This intensity can then be converted to a concentration using the sensitivity, S, of the spectrometer for the analyte considered:

$$LLD = \frac{k \times CSE}{S} \quad [11]$$

where k is a factor depending upon the confidence level chosen, S is the sensitivity (expressed as net intensity per unit concentration), and CSE is the counting statistical error of the determination. Substituting *Equation [10]* where σ_I is the CSE of the intensity of the background yields

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$$LLD = \frac{k}{S}\sqrt{\frac{I_B}{t}} \quad [12]$$

where I_B is the intensity of the background radiation at the analytical line considered. The LLD can be improved by the following:

- Increasing the sensitivity, S, of the spectrometer
- Decreasing the intensity of the background, I_B
- Increasing the measurement time, t.

The third method, increasing the measurement time, seems to be an especially easy way to improve detection limits, but it is limited because long counting times can make the method impractical. Hence, for a given spectrometer configuration, any increase in the sensitivity results in a similar increase of the background intensity. Quadrupling the sensitivity also leads to a 4-fold higher background intensity and an improvement of the LLD by a factor of 2.

3.9 Nomenclature of X-Ray Emission Lines

There are different nomenclatures in use involving the designation of the X-ray emission lines. The International Union of Pure and Applied Chemistry (IUPAC) published a systematic notation for X-ray emission lines and absorption edges, based on the energy-level designation. In practice and in many publications, Siegbahn's notation is still dominant. For the most important characteristic lines, the correspondence between Siegbahn and the IUPAC notation is given in *Table 2*.

Table 2. Correspondence between Siegbahn and IUPAC Notation for the Most Important Characteristic Lines

K-series		M-series		L-series	
Siegbahn	IUPAC	Siegbahn	IUPAC	Siegbahn	IUPAC
$K\alpha_{1,2}$ or $K\alpha$	$K–L_{2,3}$	$L\alpha_1$	$L_3–M_5$	$M\alpha_{1,2}$	$M_5–N_{6,7}$
$K\alpha_1$	$K–L_3$	$L\alpha_2$	$L_3–M_4$	$M\beta$	$M_4–N_6$
$K\alpha_2$	$K–L_2$	$L\beta_1$	$L_2–M_4$		
$K\beta_1$	$K–M_3$	$L\beta_2$	$L_3–N_5$		
$K\beta_{1,3}$	$K–M_{2,3}$	$L\gamma_1$	$L_2–N_4$		
$K\beta_2$	$K–N_{2,3}$	$L\eta$	$L_2–M_1$		
		Lt	$L_3–M_1$		

4. SAMPLE PREPARATION

With XRF it is possible to analyze most materials, irrespective of their physical state—whether they are liquids, powders, or solids—with little or no additional sample preparation. A significant advantage is the sample-size capacity: the XRF technique can accommodate large sample masses (usually in the gram weight range), thereby minimizing analytical sampling errors. This enhances the degree to which the sample presented to the spectrometer is representative of the bulk material submitted for analysis. A primary concern is that the final sample surface is flat when it is placed into the measuring position.

Measurements can be performed in a variety of environmental conditions including air, nitrogen, helium, and vacuum. Most X-ray spectrometers typically use vacuum as the medium of analysis, with the exception of some bench-top instruments. Liquid samples are incompatible with vacuum environments, so helium, air, or nitrogen at near-ambient pressure is used. Air and nitrogen readily absorb low-energy X-rays; therefore, their use is generally limited to the analysis of high-energy X-rays. Liquid and powder samples are commonly analyzed in helium, as this significantly lowers the absorption of the characteristic radiation from the specimen, compared with the effects of air or nitrogen.

4.1 Liquid Samples

Liquid samples need to be placed into disposable sample cups before being introduced to the spectrometer. The sample cup should be constructed with an appropriate supporting film known to be free of contaminant elements. Suitable films, such as polyester or polypropylene, may have a thickness as low as 1.5 µm. It is also important that the disposable cup is an appropriate size so that it is not viewed by the spectrometer; this ensures that the signals measured are coming from the sample only, and not from the sample cup.

Furthermore, it is important to understand the relationships among the sample matrix, elements of interest, and concept of infinite thickness. In general, when analyzing samples composed of low-atomic-number elements (e.g., organic matrices), it is important to use a thicker sample than when analyzing for the same elements in a heavier (e.g., metallic) matrix. This ensures that the intensity of the X-rays produced (and detected) is only dependent upon the specimen's composition and not also on the quantity of mass analyzed. The thickness at which the intensities are no longer dependent upon the thickness of the specimen presented to the spectrometer is called the *critical thickness* (also known as *infinite thickness*). The critical thickness depends upon the energies of the emission lines considered, the sample matrix, and—to a lesser degree—also on the excitation conditions. In many cases, it may not be possible to obtain infinitely thick samples because of lack of sufficient material or because the critical thickness may exceed sample cup depth; the latter can happen when analyzing liquids that are mainly composed of water or organic compounds. In these cases, correction procedures are applicable. In many cases, a simple

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modification to the analytical procedure—ensuring that all samples analyzed are of *constant* mass—is sufficient to avoid problems relating to non-infinite thickness.

An additional consideration is variation of the density between samples and standards. The analysis of samples that do not satisfy the requirement of critical thickness will be further complicated by limitations imposed by the geometry of the instrument. The X-rays incident on the sample and the X-rays emitted from the sample form a complex shape in three dimensions that is essentially a cone. The geometry is referred to as a *wedge*. This geometry limits the usefulness of traditional matrix correction because these conventional methods assume a uniform X-ray sampling volume, as opposed to a cone. In most cases, the difference between a cone-shaped geometry and the simpler geometry model is insignificant. This effect is more pronounced when measuring heavy elements in light matrix samples (such as organics). In these cases, it can have severe effects on the calculated concentrations if it is not considered. However, it is possible to include a geometric model of the wedge with a matrix correction technique referred to as the *wedge effect correction* [*7*].

An alternative solution is to have an infinitely thin layer of sample, thereby removing any possible sample interference (matrix effects), as well as the geometrical effect described above. This is not always practical, and in practice it can be difficult to prepare reproducible samples and standards.

4.2 Powder Samples

To ensure infinite sample thickness, loose powder samples may be simply weighed and placed directly into a disposable sample cup as described above. For reasons described below, it is often advantageous to lightly pack the powder, using constant pressure and a clean tool. Variations in sample compaction due to manual pressing can be compensated for by using the Compton corrections, which can account for small variations in sample thickness and/or density. These powders can also be ground to a finer particle size (if required) and then pressed to produce a solid pellet.

Preferably, all non-homogenous solid samples, such as coated tablets, should be ground using a grinding device. Similarly, any homogenous and flat sample that has too small an area to cover the instrument optical aperture should also be ground. For softer materials, a pestle and mortar may be sufficient, and for other materials swing mills, ball mills, and/or cryogenic grinding mills may be required. The analyst should ensure that the grinding means do not contribute traces of the analyte elements; this can be ascertained easily by grinding/milling pure compounds and comparing the spectra. By convention, the maximum powder (particle) size should be 50 µm or less. It is, however, more important to have a consistent particle size for both standards and samples. This ensures the homogeneity of the sample and provides an accurate representation of the entire sample, not just the near-surface layer. It is then possible to load a sample into a disposable sample cup, or to press the sample into a pellet. The disadvantage of pressing a sample into a pellet is that it may be necessary to blend a binder (e.g., wax or cellulose) into the sample. The use of such a material must be considered as a possible source of contamination. A number of different binders are available to improve the mechanical properties of the pellet. An advantage of pressing a sample is that it removes air voids, which can absorb X-rays, and thus after pressing, the measurements typically show enhanced light element reproducibility.

Furthermore, it is possible to directly measure a pressed pellet without a supporting film. It is important to note that supporting films can result in signal attenuation, depending upon the element of interest. This effect is commonly seen with lighter elements such as sodium (Na), magnesium (Mg), and aluminum (Al). The other key advantage is that the pellet ensures a flat surface that is available for reproducible excitation from all angles. In addition, pelletized samples can be measured in vacuum conditions, and this reduces any photon absorption by air or any other gas such as helium (He). Some materials such as lactose and cellulose are self-binding, thus there are no deleterious considerations when preparing a pellet except for the additional time spent on sample preparation. It is important to note that standards and samples must be treated the same from sample preparation through analysis.

Because the majority of pharmaceutical materials have an organic matrix, it is recommended to use a quantity of material corresponding to a thickness of about 1.5 cm. This can be used for both liquids and loose powder samples (provided all samples and standards have a similar matrix composition). Furthermore, any minor sample thickness variations can be compensated for by Compton ratio corrections.

5. INSTRUMENTATION

All spectrometers include a source (e.g., X-ray tube), some kind of source filtration mechanism, a sample chamber, and a detector. In addition, wavelength-dispersive systems have a set of collimators and an analyzing crystal. These components, and a few optional items, are discussed below and are further divided according to type of instrument. A diagram of a wavelength-dispersive spectrometer is shown in *Figure 2*. An energy-dispersive spectrometer is simpler in that the collimators and the crystal shown in *Figure 2* are not present, and the detector is pointed directly at the sample.

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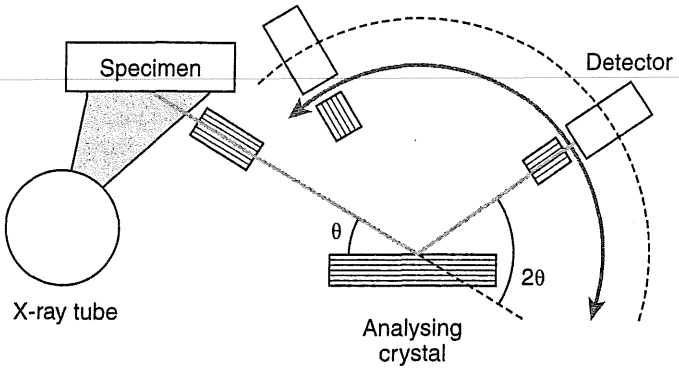

Figure 2. Schematic diagram of a wavelength-dispersive XRF spectrometer, indicating the main components.

5.1 X-Ray Tube

The two most important components of an X-ray tube are the filament (used as a cathode) and the anode. The filament is the source of electrons, which are accelerated to the anode material by a strong electric field between filament and anode. Upon impact with the anode, the electrons can gradually lose their kinetic energy, generating heat and a continuous spectrum of X-ray radiation in the process. Alternatively, when the electrons ionize atoms from the anode, the resulting vacancy can decay by emitting characteristic X-ray radiation. The total spectrum of an X-ray tube thus consists of the characteristic lines of the anode element (which is discrete in nature) superimposed on a continuum. Note that the continuum radiation, scattered by the specimen, is the main source of the background observed in XRF analysis.

5.2 Primary Beam Filter

Most X-ray spectrometers are equipped with one or more programmable beam filters. These filters are thin metal foils (typically between 50 and 1,000 microns in thickness) that are mounted on a mechanical device that can move a particular filter in the path of the primary beam. The main purpose of the primary beam is to eliminate the characteristic lines of the X-ray tube anode. If for example a rhodium anode tube is used, a significant part of the incident rhodium X-rays will be scattered by the sample. The fraction that is coherently scattered cannot be distinguished from rhodium lines that are created by atoms in the sample. In such a case, to allow for analysis of rhodium in the sample, the primary beam filter is used to prevent characteristic lines from the X-ray tube from reaching the sample. Another application of beam filters is the reduction of background in certain energy ranges. The lower limit of detection can be improved in cases where the background intensity is significant. However, the use of primary beam filters also leads to a degradation of sensitivity, and care must be taken to achieve optimum setup conditions.

5.3 Wavelength-Dispersive X-Ray Fluorescence Spectrometry

5.3.1 PRINCIPLE OF OPERATION—BRAGG'S LAW

As the name implies, the wavelength-dispersive spectrometers employ an X-ray *monochromatizing* strategy, using *Bragg's Law of Diffraction*, to split the X-ray spectrum into its individual components (or rather, narrow energy bands):

$$n\lambda = 2d\sin\theta \quad [13]$$

where n is the order of reflection; λ is the wavelength of the photons considered; d is the spacing of the crystallographic planes, and θ is the angle of incidence (and reflection) with respect to the crystal's plane. In the vast majority of applications, the first order ($n = 1$) is used. For diffraction of photons with wavelength λ to occur, the angle θ must be selected such that the product of $2d\sin\theta$ equals λ. Bragg's Law can be viewed as the requirement for positive interference of X-ray photons. Consider a monochromatic and parallel beam of X-rays of a specific wavelength λ with electrical vectors of equal amplitude in phase along any point of the direction of propagation. This beam is incident on a crystal at an angle θ. The beam is scattered in all directions. However, constructive interference only occurs in those directions for which the phase relationship is conserved. This happens at an angle θ for incident rays 1 and 2 (see *Figure 3*), for which the path difference $AB + BC$ is equal to an integer number of wavelengths λ. The integer n refers to the order of the diffraction. The most intense diffraction peaks—and thus the highest measured intensity—can be obtained when $n = 1$ (first order). Higher-order diffractions with $n = 2$, 3, or 4 do occur, but the efficiency is low.

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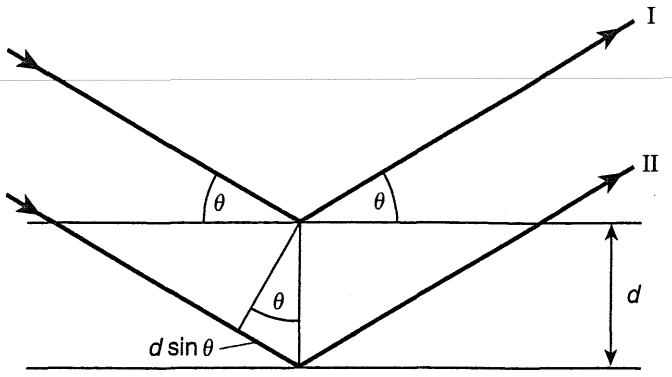

Figure 3. Geometry involving Bragg's law.

5.3.2 DISPERSION

The *dispersion* of a WDXRF instrument can be calculated by differentiating *Equation [13]* as follows:

$$\frac{d\theta}{d\lambda} = \frac{n}{2d\cos\theta} \quad [14]$$

Dispersion is a measure of the angular separation between peaks corresponding to two different wavelengths. A larger value for the dispersion indicates a larger angular separation, thus a smaller (potential) overlap between photons of slightly different wavelengths. Decreasing the *2d* spacing is an obvious way to improve on angular dispersion; the *2d* spacing is, however, determined by the crystal used. For most of the wavelength range, there are at least two crystals available; the one with the smaller spacing offers the better angular separation, but typically this involves a loss of intensity. Well-defined crystals with known *2d* spacing are used as monochromators. A list of commonly used crystals appears in *Table 3*.

Table 3. Commonly Used Monochromators in WDXRF

Crystal	Name	Reflection Plane	*2d* Spacing (nm)	Element Range
LiF(220)	Lithium fluoride	(220)	0.2848	V-U
LiF(200)	Lithium fluoride	(200)	0.4028	K-U
Ge	Germanium	(111)	0.6532	P, S, Cl
PE or PET	Pentaerythritol	(002)	0.8742	Cl-Al
TlAP	Thallium acid phthalate	(1010)	2.575	Mg-O
	Layered synthetic microstructure		3–12	Mg-Be

Dispersion only describes the *angular separation* between two peaks; it does not describe the width of the peak, which is typically referred to as resolution (in other forms of spectrometry) and is calculated from the full width of the peak at half height. The actual resolution from the detector system has no discernible influence on peak separation.

The choice of crystal and angle of the detector relative to the crystal determine which elemental X-ray lines may enter the detector. The main task of the detectors in WDXRF is reduced to counting photons with a known energy, as the use of the monochromator crystal thus separates the selection of the wavelength of the photons from the actual counting.

5.3.3 DETECTORS FOR WDXRF

Gas-filled detectors: Gas-filled detectors consist of a cylindrical volume filled with a noble gas such as argon (Ar) or krypton (Kr), and a wire running through the center of the detector (see *Figure 4*).

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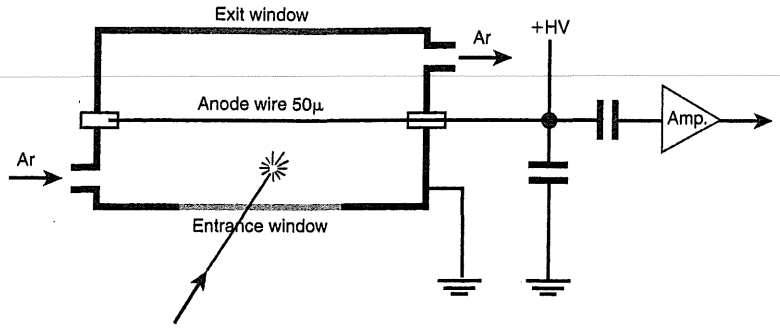

Figure 4. Schematic diagram of a flow counter.

An incoming photon (represented by its energy $h\nu$) can remove an outer (valence) electron of one of the inert gas atoms. For an argon-filled detector:

$$Ar \xrightarrow{h\nu} Ar^{+} + e^{-} \quad [15]$$

The energy required for the ionization, e_i, depends on the element; for argon (Ar), about 26 eV is required to create an ion-electron pair. The total number of such primary ionizations, n_p, caused per incident photon with energy, E, can then be calculated using

$$n_p = \frac{E}{e_i} \quad [16]$$

The electric field between the wire and the outer body will cause the electrons to accelerate toward the anode wire. The positive ions, on the other hand, will migrate toward the housing. The resulting electric field gives rise to a cascade of ionization events. The result of this cascade effect is as follows: for each of the individual electrons originally created by the incident photon, many more electrons are finally collected at the anode wire (this is called the *gas amplification factor* and is typically around 10^4 to 10^6), creating a voltage drop that is then processed by the counting electronics.

The entrance window should absorb as few of the incoming photons as possible, because any photons absorbed into the window are lost from the counting circuit, thus reducing the sensitivity. Large windows with low attenuation for long wavelengths can be made from stretched polymers, such as polyethylene or polyester, coated with a conductive material at the interior of the counter to provide for a homogeneous electric field. Such thin windows need a mechanical support to withstand the pressure difference caused by the vacuum in the spectrometer chamber. The collimator is usually preferred as a support because it will also reduce the amount of radiation that is scattered from the crystal or the multilayer.

On the other hand, the polymer windows have small pinholes. These pinholes allow the counting gas to gradually leak away. Such detectors would have only a very short lifetime before the counting gas leaked away or became so contaminated that it would effectively render the detector useless. To compensate for the leaks, a constant flow of counting gas is used, the excess of which is vented away out of the spectrometer chamber; such detectors are therefore called *flow proportional counters* (or gas flow detectors). Only a fraction of this flow is leaking into the spectrometer chamber, whereas the remainder is leaving the detector through a tube and is vented into the atmosphere. It is essential for the overall stability of the spectrometer that the pressure and temperature of the gas are kept constant. Hence, most instruments are equipped with a gas-density stabilizer.

Gas flow proportional counters are the preferred counters for the detection of X-rays with very long wavelengths in wavelength-dispersive spectrometers. The counting efficiency of the gas-filled detectors is determined by the absorption in the entrance window and the capture efficiency (absorption of the incident radiation) of the counting gas for the wavelengths of interest. The capture efficiency depends on the path length available within the detector, the composition of the counting gas, and its pressure. The absorption properties of the window determine the efficiency of the detector at the long wavelengths, whereas the absorption properties of the gas determine the efficiency at the shorter wavelengths. Considering the typical dimensions, the nature of the gas, and the pressure, the absorption of the gas as function of the wavelength of the incident photon can be calculated. Around wavelengths of 0.2–0.3 nm, about half of the incident photons are not absorbed, reflecting a low efficiency. This is the main reason why sequential WDXRF instrumentation is equipped with more than one detector.

Scintillation counter: WDXRF instruments are generally fitted with a scintillation counter for the detection of shorter wavelengths for which the gas proportional counter is less efficient. The operation of the scintillation counter is based on two distinct stages: a scintillation crystal and a photo-multiplier tube (see *Figure 5*).

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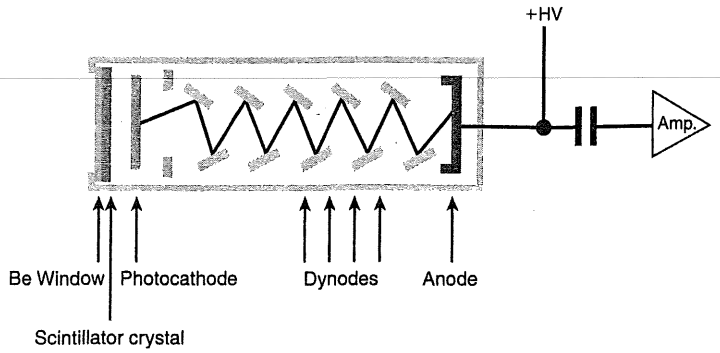

Figure 5. Schematic diagram of a scintillation counter.

In the first stage, the incoming photon is absorbed by a "scintillator crystal". A scintillator is a material that emits light when it is excited by ionizing radiation, such as X-rays. Many different scintillator materials exist, with different properties regarding radiation hardness, operating temperature range, decay times, optical properties, and other properties. The most commonly used scintillator material in WDXRF instruments is a sodium iodide (NaI) crystal, doped with thallium (Tl). The scintillating crystal is optically coupled to a "photo-multiplier", which consists of a photo-cathode and a series of dynodes. In the second stage, upon impact by a photon, the photo-cathode releases free electrons. These are then accelerated toward a series of dynodes. At each dynode, the impact of the electrons generates more electrons. If 10 dynodes are used, the total amplification provided is about a factor of 10^6.

The counting efficiency of the scintillation counter is largely determined by the thickness of the sodium iodide thallium [NaI(Tl)] crystal. The counting efficiency for a 3-mm-thick crystal is better than 99% for wavelengths of 0.05 nm and longer. This allows the detection of high-energy photons such as Sn Kα with high efficiency.

The production of light photons is higher in the scintillation detector than the number of electron-ion pairs created in a flow counter; this should lead to a better resolution. However, this advantage is cancelled out by the low yield of photo-electrons by the photo-cathode, where less than 1 electron is freed for every 10 incident light photons. Compared to the gas-filled counters, the resolution of a scintillation counter is worse.

The use of the WDXRF technique (typically with power in the kW range) for organic matrix sample analysis is limited because heat generated by the X-rays may be sufficient to induce significant specimen alteration, such as browning or burning of the sample surface. Furthermore, even if the sample is measured for a very short time by WDXRF, the heat imparted to the sample surface may induce the loss of volatile elements [e.g., mercury (Hg) and selenium (Se)]. The WDXRF technique is, however, perfectly suited to measure inorganic materials that can withstand elevated temperatures. Examples of common inorganic materials that are suitable for WDXRF measurement are calcium carbonate ($CaCO_3$) and sodium chloride (NaCl).

5.4 Energy-Dispersive X-Ray Fluorescence Spectrometry

5.4.1 PRINCIPLE OF OPERATION

The energy-dispersive XRF technique typically involves simultaneous detection of photons of different energies that are emitted by the atoms in the sample. The detector determines both the photon's energy and the intensity, which is the number of photons per unit of time. To provide adequate resolution for distinguishing between characteristic lines and overlapping lines from other elements, most EDXRF instruments are equipped with a high-resolution detector and a multi-channel analyzer (MCA). The MCA essentially provides a histogram representation of the spectrum. High-end instruments use sophisticated calculation (deconvolution) algorithms to identify the peaks (i.e., to establish the presence of certain elements) and to obtain intensity data from the histogram. EDXRF is theoretically a simultaneous technique because it measures the complete spectrum in a single counting phase. However, significant improvements in performance can be achieved by measuring the sample using two or more different excitation conditions, each optimized for a certain energy range.

Compared to WDXRF, the design of an EDXRF spectrometer is much simpler because it lacks a high-precision goniometer and the associated collimators. The positioning of the detector relative to the sample is very simple; the most obvious requirement is that there should not be a direct path between the anode of the X-ray tube through the entrance window of the detector onto the active body. Distances between the X-ray tube window and specimen, and between the specimen and detector entrance window, can be small, in the order of 1–2 cm. Furthermore, as only limited collimation is required, the angle of acceptance of the photons from the specimen can be quite large. Low-power X-ray tubes with ratings up to 50 W are used. Because of the inherent simplicity of their design, wide variation of systems with varying capabilities exists. A typical spectrum on an energy-dispersive spectrometer is shown in *Figure 6*. The energy on the horizontal axis increases from left to right. The peak shape is nearly perfect Gaussian.

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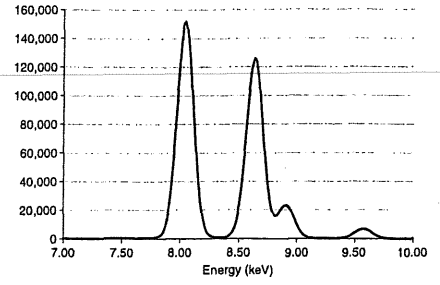

Figure 6. Energy-dispersive spectrum of a brass specimen. From left, the peaks are due to Cu Kα, Zn Kα, Cu Kβ, and Zn Kβ. The horizontal axis is energy and the vertical axis is in counts per channel. This spectrum was recorded at 75,000 cps with 50 kV and an Ag primary filter to reduce the background. The full width at half maximum (FWHM) is about 165 eV at the Cu Kα peak.

5.4.2 DETECTORS

The resolution of the detector system determines both the peak width (expressed as FWHM) and the peak separation. Systems may be equipped with gas-filled, solid-state, or semi-conductor detectors. The three most common solid-state detectors used are the following:

- Liquid nitrogen-cooled silicon-lithium drifted Si(Li)
- Liquid nitrogen-cooled germanium detectors, such as the LeGe and HPGe
- Detectors based on intrinsic silicon, such as the PIN (positive, intrinsic, and negative) detector and the SDD (silicon drift detector).

The first two types of detectors allow for high photon-capture efficiency but require relatively bulky, well-insulated containers (Dewar) for the liquid nitrogen. These detectors are generally rather large, which improves sensitivity, and thick, which improves capture efficiency for high-energy photons. They are typically found on floor-standing equipment. Bench-top systems are equipped with detectors that are based on intrinsic silicon. These types of detectors do not require cooling to temperatures as low as the other two categories; temperatures between −25° and −15° are acceptable. Peltier-cooling or electro-thermal cooling is sufficient to reach those temperatures. The detectors are smaller and thinner than their bulk siblings. Older equipment might still be using so-called PIN detectors, but all of the newer equipment tends to be equipped with an SDD. The SDDs have much better resolution and can be used at much higher intensities than the PIN detectors, which are typically limited to about 220 eV at Mn Kα. The SDDs combined with state-of-the-art electronics have a resolution (expressed as FWHM) of better than 145 eV at Mn Kα. This is comparable to liquid nitrogen-cooled semi-conductor detectors. A word of caution is required as the resolution worsens with increasing count rate or intensity. Most manufacturers therefore specify resolution at an impractical, low count rate of 1,000 or 2,000 cps.

5.5 Polarized X-Ray Spectrometry

5.5.1 PRINCIPLE OF OPERATION

The most elaborate EDXRF spectrometers use polarization or a secondary target to modify the spectral distribution of the X-ray source; the aim is to reduce, or ideally eliminate, the intensity of the continuum background. This effort is a logical consequence of *Equation [3]*. Due to the fact that the major component of the background is scattered tube radiation, one method of preventing radiation from the X-ray tube from reaching the detector system is the use of polarized radiation. These systems have applications in the analysis of, for example, residues of palladium (Pd) catalyst in some pharmaceutical ingredients.

5.5.2 POLARIZED RADIATION AND POLARIZING TARGET

The orientation of the electric field vector, E, of the photons generated in an X-ray tube is random but always perpendicular to the direction of propagation. Using a Cartesian coordinate system, with the z-direction coinciding with the direction of propagation, the two components of the electric field vector in the x-direction (denoted by E_x) and y-direction (E_y) are identical and obey the following relationship:

$$E_x^2 = E_y^2 = \frac{1}{2}E^2 \quad [17]$$

The component along the axis of propagation, E_z is zero; this is because electromagnetic radiation is a transverse wave. After *coherent scattering* in the yz plane, the field vector of photons traveling in the y direction, E_y, is also reduced to zero. This beam of photons, all with their electric field vector parallel to the x-direction, is directed at the sample and interacts in the usual way. The photons cause ionizations and can be scattered, but they cannot be scattered in the direction along the x-axis, because the electric field vector cannot have a component parallel to the direction of propagation. If the detector is now placed somewhere along the x-direction (still in the same frame of reference), the background is significantly reduced because the main contribution (i.e., scatter from the incident radiation) is absent. The process is inefficient because there is no active polarization; it is entirely achieved by suppressing one component of the electromagnetic wave, thereby significantly reducing its intensity. Therefore, high-power X-ray tubes are used.

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5.5.3 SECONDARY TARGETS

The use of secondary targets in energy-dispersive spectrometry has been widespread for high-power instrumentation. The aim is to reduce the background, thus improving the lower limit of detection. When *electrons* decelerate, they generate Bremsstrahlung of a continuous nature; however, photons do not generate Bremsstrahlung. The principle behind an instrument equipped with secondary targets is to use a classic X-ray tube to provide a beam of X-rays (including the troublesome continuum) and direct it at a secondary target, which is typically a metal. The radiation emanating from the secondary target will consist of the very intense characteristic radiation corresponding to the nature of the target and scattered continuum. The intensity of the scattered background is relatively low, especially when the target material is a metal of medium-to-high atomic number. There is no additional continuum created by the incident photons, but a tiny contribution to background radiation is created by the high-energy photo-electrons. The radiation from the secondary target is directed at the sample for excitation purposes. The spectrum from the sample is composed of the characteristic lines from the elements present, the scattered characteristic radiation from the secondary target, and the concomitantly scattered continuum from the tube. The latter contribution is very weak because it has been subjected to two scattering events, one at the secondary target and another one at the sample. If the instrument is equipped with a selection of secondary targets, then significant improvements in the lower limit of detection can be obtained. The two-step process requires a higher-power X-ray tube to get the same flux at the sample when compared to direct excitation, so it is generally not incorporated into bench-top instrumentation. Currently, secondary targets are often used in combination with polarization targets. This combination is straightforward, because both types of targets can use the same geometrical arrangement.

5.6 Total Reflection X-Ray Fluorescence Spectrometry

Another variety, *total reflection X-ray fluorescence* (TXRF, also sometimes abbreviated to TRXRF), specializes in the analysis of infinitely thin and extremely homogenous samples derived from liquids. Typically, TXRF instrumentation employs an X-ray source, which is directed at the sample under a very small angle with respect to the surface. The detector is typically placed above the sample at a 90° angle. The TXRF technique commonly uses internal standards (mixed into the unknowns) and geometrical relationships to determine elemental concentrations.

6. QUALITATIVE XRF ANALYSIS

This section applies to both WDXRF and EDXRF.

6.1 X-Ray Spectra

Qualitative analysis by means of X-ray fluorescence is relatively simple because the characteristic line spectra of the elements are simple and are not affected by chemical bonding; i.e., these spectra are truly elemental. For qualitative (and quantitative) analysis, the main lines are those from the K- and L-series. The K-lines result from electron transitions after one of the K-electrons has been expelled. Due to quantum mechanical selection rules, not all transitions are allowed. Transitions from the L-shell to the K-shell are limited to transitions involving L2 and L3, as radiative transitions between the L1-shell and K-shell are forbidden. These transitions are the most probable, leading to the most dominant line in the spectra. For most elements, the energy difference between the L2-shell and L3-shell is rather small (in the order of a few eV), so the emitted lines are typically not separated and simply considered a doublet. Radiative transitions from M- and N-shells to the K-shell are also occurring. These give rise to a more complex part of the K-spectrum. In practice, however, only the $K\beta_{1,3}$ doublet (resulting from transitions between M3 and K and M2 and K) is of importance. The relative intensity between the Kα and the Kβ varies from 1 in 1,000 for sodium (Na) to 1 in 6 for plutonium (Pu), with the Kα always being the most intense line of the series.

The Kα-line is generated by an electron transition from an electron in the L3 shell, following the removal of a K-shell electron. The Kα-line is the most dominant line in an element's series. The K electrons of an element, however, have the highest binding energy of all electrons in the atom, and thus are only excited by high-energy incident photons. This is generally not a problem for the low- and middle-atomic number elements, but excitation of the K-lines of elements with atomic numbers above 50, for example, might not be possible, as the excitation potential is higher than the maximum operational voltage on most X-ray tubes.

Whereas the K-line spectra are typically limited to two lines only, the situation for the L-series spectra is more complicated. For lead (Pb), a total of 46 L-lines are listed in Bearden [*2*]. Many of these are weak, or are doublets. For qualitative analysis, not all of these lines are to be considered. In all cases, it is important to realize that if the Kβ is visible, then the Kα must be visible too and must have a higher intensity.

6.2 Qualitative Analysis

Qualitative analysis typically consists of a few simple steps. After the spectra have been collected, the background is subtracted. This background subtraction can be done in a separate step, or it can be done in conjunction with a peak search algorithm. A fraction of these photons propagate in the direction of the analyzing crystal (WDXRF) or directly to the detector (EDXRF). Next, a peak search is performed, and finally, these peaks are identified, i.e., each is attributed to a particular element. Efficient algorithms for qualitative analysis look for the presence of each element, rather than trying to identify each peak. This is different from X-ray diffraction, where each peak is checked for a match with a phase. In this method, the peak search is performed first. Then, for each element, the presence of the $K\alpha_{1,2}$-doublet is verified. If this line is found in the spectrum, then the weaker lines of the same series ($K\beta_{1,3}$ and $K\beta_2$) are investigated. Note that all characteristic lines in the same series should appear, except in cases where the intensity of a weaker line is similar to the intensity of the background. For elements with

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higher atomic numbers where the K-lines are not excited (or not detected due to limitations in detection hardware) the process starts with the L-line spectra. The intensity ratios of, for example, Kα to Kβ are listed in several databases [*8,9*]. The tabulated ratios are for the "isolated" atom; they reflect the intensities as obtained from an atom without any matrix effects. These ratios can be corrected quite easily for matrix effects, even in the case of overlapping lines. Only elements that are present at trace levels (with intensities comparable to the standard deviation of the background intensity) are usually deemed "not present". Note that, in principle, line interference between elements is not much of a problem in qualitative analysis; this is due to the fact that in qualitative analysis, many more data points that cover alternative lines are available. Hence, establishing the presence of elements is not impacted as much as one would suspect. Also, in energy-dispersive spectrometry, where the line overlap is most severe, adequate corrections can be performed because the peak shape is rather well known.

7. QUANTITATIVE XRF ANALYSIS

7.1 Selection of Analytical Line

The characteristic emission spectra of most elements contain several lines. The choice of an analytical line for a given element is largely dependent on three factors: the concentration of the element in the sample, the potentially interfering lines, and the excitation capabilities of the spectrometer. In most cases, the Kα emission line of an element is preferred. When the excitation of the K-lines is not possible due to limitations of the tube and/or generator, the use of L-lines, with their lower energy, is necessary. Use of the Lα-line (when the Kα-line is not excited) does not involve much of a detriment because for those elements, the intensity of the L-lines is quite high. Also, the detection efficiency of the L-line photons is then much higher than that of the high-energy K-lines of the same element. For elements with high atomic numbers (i.e., Z larger than about 72), the Lβ offers a very good alternative to the Lα and these lines have comparable sensitivities.

In general, on a typical WDXRF instrument, all elements with atomic numbers up to 56 are measured on their Kα-line. For elements with higher atomic numbers, the Lα-line is used. For EDXRF spectrometers equipped with SDD-using generators (and tubes) with a maximum voltage of at least 30 kV, K-lines are available to measure for elements up through about Z = 47; the L-line spectra are used for all elements with higher atomic numbers. The exact point at which the switch over occurs between the K-line and the L-line spectra depends on factors such as the application and the anode material of the X-ray tube used.

Quantification procedures are carried out using routine spectroscopic techniques involving the measurement of blanks and standards of varying elemental concentrations. The measured intensities are then corrected for background and for line overlap, if required. In a number of cases, a straightforward linear relationship between the intensity of the analyte and its concentration can be established. This is especially the case with low concentrations in a constant matrix. In the more general case, however, corrections for matrix effects are required. Matrix effects are not the limiting factor in the precision of the final analysis, but they must be accounted for in order to obtain an accurate measurement.

7.2 Matrix Correction Techniques

7.2.1 ORIGIN OF MATRIX EFFECTS

XRF is typically applied to condensed samples, without resorting to dissolution of the sample in an abundance of acids or other solvents. Thus, the samples remain concentrated, in contrast to many other techniques. Because of this, the XRF technique is prone to matrix effects, in that the relationship between the intensity of a particular analyte and its concentration is generally not as simple and straightforward as with diluted specimens. With the latter, this relationship between concentration and intensity is often expressed as a straight line. However, the presence of other elements at high concentrations can cause deviations of this straight-line relationship. The physics of X-ray emission and detection is well understood and relatively simple for bulk materials, and this provides a solid theoretical basis for a mathematical description of matrix effects. Matrix effects can thus be described accurately and correctly using a variety of mathematical techniques, and several methods have been developed to address them. Two main matrix effects can be distinguished and are discussed below—absorption and enhancement.

Absorption: Absorption as a matrix effect occurs when the matrix elements and the analyte element have different absorption properties for the characteristic radiation of the analyte. If the mass attenuation coefficient of one or more of the matrix elements is larger than the mass attenuation coefficient of the analyte, the matrix will absorb more of the analyte radiation than expected and the measured intensity of the analyte will be lower. This results in a concave calibration curve. If, on the other hand, the absorption of the matrix elements is lower, a higher intensity of the analyte will be measured and a convex calibration curve will be observed. Absorption as a matrix effect can be large, reducing the intensity by as much as 100% (and more) or "increasing" it by as much as 10%, depending upon the analyte and matrix elements. The magnitude of the absorption effect depends on the wavelength of the analytical line considered and the composition of the specimen. In a given specimen, the absorption effect can be significant for the characteristic line of one analyte and rather insignificant for another. The absorption effect can also differ significantly for the same analyte when measured on two different characteristic lines.

The three processes that cause absorption are the photo-electric effect, the coherent scatter, and the incoherent scatter. In general terms, photons with a given energy will be absorbed more with increasing atomic number of the absorbing element. Also, the absorption by a given element decreases in a steady, smooth manner when the photon energy increases. However, when the photon energy exceeds the binding energy of electrons in an inner shell, the photo-electric effect (see *3.1 Ionization and the Photo-Electric Effect*) causes an increase in absorption. This leads to discontinuities in the absorption curve at energies corresponding to the binding energy of the electron levels. These discontinuities are called absorption edges; the energy at which the absorption edge occurs is equal to the binding energy of the electron shell concerned.

Enhancement: *Enhancement* as a matrix effect is the process whereby the characteristic radiation of matrix elements is absorbed by atoms of the analyte, subsequently causing characteristic radiation of the analyte. The intensity of the analyte is thus enhanced, which leads to concave calibration curves. For a given analyte, all characteristic lines from the same series are

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enhanced to the same degree. This is because the root cause of the enhancement is the photo-electric absorption of radiation from matrix elements. The effect of enhancement is typically smaller than that of absorption. It is most pronounced in alloys of the first-row transition elements, where it can reach 25%. In many other applications, the enhancement effect is smaller, but is still important enough to warrant consideration and correction.

7.2.2 FUNDAMENTAL PARAMETER METHODS

The physical processes involved in absorption and excitation in XRF are well understood and easy to model mathematically. All entities required are listed in a variety of tables and databases. The calculations were previously tedious and time consuming, but with the current computing power available, the calculation effort is no longer an obstacle. Fundamental parameter methods are based on the underlying physical processes governing X-ray fluorescence, including the specifics of the instrument's geometry, the anode material of the X-ray tube, the voltage on the tube, and the composition of the specimen. The derivation of the equations is beyond the scope of this chapter, but is addressed by Criss and Birks [*10*] and Shiraiwa and Fujino [*11*].

7.2.3 COMPTON CORRECTION

The use of Compton scattered radiation as a means to correct for matrix effects (as well as for variations in sample preparation) has been described by Reynolds [*12*] and Taylor and Andermann [*13*]. The Compton correction is based on the fact that the intensity of incoherent (or Compton) scatter is inversely related to the mass attenuation coefficient of the sample. In practice, a high-energy characteristic line from the tube anode is used. Measuring the intensity of the Compton scattered tube line thus reveals information about the absorption properties at the wavelength of the Compton scattered line. At first glance this seems to be of limited use, but in the field of X-rays, values of the mass attenuation coefficients for the same sample at different energies/wavelengths are proportional. This relationship, and the fact that absorption is the major source of matrix effects, explains why the Compton correction is a powerful correction method in a variety of matrices.

It is, however, not generally applicable and will fail in cases where there are significant absorption edges between the wavelengths of the Compton scattered radiation, at the wavelength of the analytical line used. The correction is based on the relationship between Compton scatter and the sample's absorption properties, and it does not correct for enhancement.

7.2.4 INFLUENCE COEFFICIENT ALGORITHMS

Matrix correction methods based on influence coefficients have the general format:

$$C_i = K_i I_i \left[1 + \sum_{\substack{j=1 \\ j \neq i}}^{n} \alpha_{ij} C_j \right] \quad [18]$$

(based on Lachance and Traill [*14*]) where C_i and C_j are the concentrations of the analyte i and the interfering element j, respectively, and α_{ij} is the influence coefficient, expressing the influence of interfering element j on the analyte i. All concentrations are expressed as weight fraction. The summation in *Equation [18]* has $n - 1$ terms, for a sample consisting of n elements or compounds; this is a simple yet essential feature of the algorithm. The same set of equations with n terms in the summation would lead to a homogeneous set of simultaneous equations, from which only *ratios* rather than absolute values could be derived.

Because the sum of the concentrations of all elements (whether they are measured or not) in a specimen always totals 1, one element can be eliminated. Most influence coefficient algorithms in software are based on this equation, however they may differ in *how the values of the coefficients are calculated.*

7.2.5 EMPIRICAL INFLUENCE COEFFICIENTS

Lachance and Traill [*14*] indicate that the value of the coefficients should be calculated from theory, but they only provide a simple and limited method that fails to consider enhancement or the polychromatic nature of the incident beam. Therefore, in many cases the coefficients were determined using multiple regression methods. Many early variations on influence coefficient algorithms were based on regression techniques that were used to calculate the value of the coefficients. However, the use of regression methods should be discouraged as they often lead to overly optimistic indications of precision and then often fail during validation. This is especially problematic if not enough certified reference materials are used in the calibration. The minimum number of standards used should be at least 3 times the number of parameters. Each of the influence coefficients whose value is determined by regression consumes one degree of freedom, as does the determination of slope and intercept.

The total number of parameters to be determined can grow rapidly for a single analyte. For example, assume that there are three interfering elements, and thus with slope and intercept, a total of five parameters need to be determined. This requires a minimum of 15 *independent* and *uncorrelated* standard samples. Note that replicates of the same standard sample do not count as individual samples, but instead count as a single one; this is a common source of error. Also, care must be taken to ensure that the standards are uncorrelated. This implies that a series of dilutions from a given standard cannot be used when empirical influence coefficients are to be determined. In a dilution series, two (or more) components *A* and *B* increase (or decrease) in the same direction. Thus the effect of component *A* on the intensity of the analyte cannot be distinguished from component *B*'s effects, leading to erroneous values for the influence coefficients.

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7.2.6 THEORETICALLY CALCULATED INFLUENCE COEFFICIENTS

De Jongh [*15*] was the first to publish a method detailing the calculation of influence coefficients, using theory only to eliminate a compound other than the analyte. The algorithm is still in common use today. The eliminated compound can be the non-fluorescing matrix, such as cellulose, or the matrix when its concentration is not certified. Note that the concentration range over which the algorithm delivers precise results is rather limited when the matrix effects are severe. This is a direct consequence of the fact that the values of the influence coefficient are calculated for a particular composition of the sample. For routine analysis involving relatively limited concentration ranges, the algorithm delivers excellent results. In contrast to the situation where the values of influence coefficients are determined by regression (see *7.2.5 Empirical Influence Coefficients*) it is feasible to calibrate using dilution series.

7.3 Measurement Method Development and Calibration

7.3.1 OVERVIEW

Once the elements of interest have been identified, the spectrometer measurement parameters must be set appropriately. This involves determining the "ideal" measurement conditions for each element. The list of parameters to be selected for each analyte can include, for example, the voltage and current on the X-ray tube, the crystal and the collimator (WDXRF), the type of primary beam filter (if used), and other parameters. For elements that are similar in atomic number, the settings will be quite similar. The precision of XRF measurements is theoretically limited by the CSE. It has been shown that the CSE of a measurement of N counts (photons) is given by the square root of N. It is a generally accepted practice to determine the measurement times that obtain an acceptable CSE for each element of interest. Sample preparation errors and the CSE are the largest contributors to the total analytical precision. Once the measurement time has been established, it is important to ensure that the background positions and methods for peak fitting are also appropriate.

After the net intensities of the elements of interest have been obtained from a suitable number of reference standards, a calibration can be performed. For low concentrations of analytes in an otherwise constant matrix, linear regression may be appropriate. When evaluating specimens with higher concentrations of analytes, the calibration also involves correction for matrix effects. The fact that the EDXRF technique is not capable of measuring the organic constituents of an actual pharmaceutical composition is of little concern. This is certainly true if the composition of the matrix is constant, and if the total content of measurable elements is low. But if these two conditions are not satisfied, the difference in scattering properties can result in calculation errors if not corrected. Fortunately, there are numerous strategies that accommodate these differences, such as the fundamental parameters method or the Compton correction method. The nature of matrix effects, as well as methods to correct for them, have been discussed above. The application of these methods to calibration and analysis of unknowns will be discussed in the sections below.

7.3.2 CALIBRATION USING INFLUENCE COEFFICIENT ALGORITHMS

Calibration using influence coefficients is rather straightforward. Once the values for the coefficients have been calculated for each of the standard samples, the summation

$$M_i = \left[1 + \sum_{\substack{j=1 \\ j \neq e}}^{n} \alpha_{ij} C_j\right] \quad [19]$$

can be calculated where for standard samples, the concentrations, C_j, are known. The value of M_i for one standard sample will be different from the value for another standard sample, and it does not matter whether the difference is significant or slight (large or small). If adequate corrections for background have been made, calibration is then represented by

$$C_i = K_i I_i M_i \; [20]$$

where the product of the measured intensity, I_i, and the corresponding value for the matrix effect, M_i, are regressed against the given, known concentration, C_i, to determine the slope, K_i. This equation assumes that the intensity, I_i, is properly corrected for the background. In those cases where the intensity of the background is constant between samples and standards, a slightly modified equation can be used:

$$C_i = B_i + K_i I_i M_i \; [21]$$

where B_i is the intensity of the background. In this case, both B_i and K_i are determined by regression analysis. In situations where the background intensity varies significantly between samples, or between samples and standards, proper background correction must be applied to the measured intensities, and the corrected intensities must be used for the calibration.

7.3.3 CALIBRATION USING COMPTON CORRECTION

In this case, both the intensity of the analyte, I_i, and the intensity of the Compton scattered line, I_C, are measured. The intensity of the Compton is inversely proportional to the mass attenuation coefficient [*12*], or

$$M_i = \frac{1}{I_C} \quad [22]$$

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Combining this with *Equation [20]* leads to

$$C_i = K_i I_i \frac{1}{I_c} \quad [23]$$

The intensity of the analyte and the Compton line are measured on a suite of standard samples and regressed against the analyte concentration to determine the value of the slope of the calibration. A background factor, B_i, can be determined as well.

7.3.4 CALIBRATION USING FUNDAMENTAL PARAMETER METHODS

The physical processes involved in absorption and excitation during XRF are well understood, and it is possible to calculate the value of the matrix effect based on several factors: the composition of the sample, the type and settings of the tube, the geometry of the spectrometer, and the fundamental parameters:

$$M_i = f(C_i, C_j \ldots, C_n, \textit{tube}, \textit{geometry}, \textit{fp}) \quad [24]$$

This value of M_i is calculated for each standard and for each analyte of interest, and then applied in the usual manner.

7.3.5 INTERNAL STANDARD METHOD

An alternative to the methods described above is the internal standard method. A fixed quantity of an element *s* (other than the analyte *i*) is added to all specimens. The element *s* is chosen such that it is not present in the specimens, thus ensuring that its concentration is the same for all specimens. In the absence of matrix effects, the measured intensity of its characteristic radiation would also be constant. Any variation in its intensity (other than the variation resulting from the counting statistical error) is attributed to the presence of matrix effects. The idea is to choose the internal standard element *s* in such a way that the intensity of its characteristic radiation is subject to the same matrix effects as the intensity of the analyte element under consideration. Both intensities—of the analyte and of the added element—increase (or decrease) in proportion to one another depending upon the matrix effect. *Equation [22]* can be modified for the internal standard element *s*:

$$C_s = K_s I_s M_s \quad [25]$$

In this equation, C_s is a constant. Taking the ratio of *Equation [20]* and *Equation [25]* and solving for the only unknown, C_i, yields

$$C_i = \frac{K_i M_i C_s}{K_s M_s} \frac{I_i}{I_s} \quad [26]$$

It is important to note that the terms of the first fraction in *Equation [26]* are either all constants (K_i, K_s, and C_s) or are proportional to one another (M_i/M_s). *Equation [26]* can thus be reduced to

$$C_i = K_i^* \frac{I_i}{I_s} \quad [27]$$

where all of these constants are collated in a single variable K_i^*.

The main challenge in applying this method is the selection of the element to be used as the internal standard. This element must not be present in the specimens (or at most, present at very low levels that will not interfere with the analysis). Also, its characteristic radiation must be subject to the same matrix effects as the analyte(s) of interest, and its emission spectrum should not cause interference. In particular, care must be taken to ensure that the analyte element and the internal standard are both subject to (or both *not* subject to) enhancement by a matrix element. In the same fashion, when the analyte is in the presence of matrix elements that strongly absorb the characteristic radiation of the analyte, the same should apply to the characteristic intensity of the internal standard element.

When the samples are organic matrices typical of pharmaceuticals, it is easy to satisfy the essential requirement that the characteristic radiation of both the analyte(s) and the internal standard are subject to the same matrix effects. These organic matrices do not exhibit characteristic radiation of their constituent elements, which makes it easier to find a suitable internal standard element. A simple guideline is to choose an element that is not present in the samples and that has an emission line close to the one of the analyte's (with regard to either wavelength or energy). The characteristic lines themselves do not have to be the same or even from the same series, and the method also works when the analytical line is a Kα and the line of the internal standard is, for example, an Lβ. It must be noted that there is no requirement for the internal standard element to be a pure element. Stable compounds that are easily soluble in a solvent can be used. The solvent is, in many cases, the same as the base material of the specimens and the calibration standards. If more than one internal standard element is to be used, it is recommended that both elements/compounds are added in a single internal standard solution, to minimize errors.

7.4 System Suitability Criteria

Performance characteristics that demonstrate the suitability of an XRF method are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Validation of Compendial Procedures* ⟨1225⟩.

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7.4.1 LIMIT OF DETECTION AND LIMIT OF QUANTITATION

The lower limit of detection may be expressed as:

$$LLD = \frac{3.3 \times CSE}{S} = \frac{3.3 \times C}{I_{net}} \sqrt{\frac{I_b}{t_b}} \quad [28]$$

with I_{net} representing the net count rate at the concentration, C; I_b as the count rate of the background; and t_b as the background measurement time. It is noteworthy that the detection limit will improve with the square root of the measuring time.

The limit of quantitation can be estimated by calculating the standard deviation of NLT six replicate measurements of a blank and multiplying by 10. Other suitable approaches may be used (see ⟨1225⟩).

7.4.2 LONG-TERM STABILITY AND DRIFT CORRECTION

Under standard operating conditions, instrumental drift of X-ray spectrometers is less than 1% relative and can be as small as 0.1% relative for high-end instrumentation in well-maintained laboratories over the same time period. To monitor instrument drift, one or more stable sample(s) with reasonable intensities (to minimize the CSE) for the analytes of interest can be measured on a regular basis. It is not necessary to use *in-type* drift correction samples. It is common to use glassy samples and alloys. Most instruments have drift correction routines within the software, and because XRF is a very robust technique, it may only be necessary to utilize a drift-monitor once per month. However, it is recommended that most laboratories practice drift monitoring more frequently.

7.4.3 ANALYSIS

Once a method has been established through the calibration procedure, a sample analysis may be performed. It is essential to ensure that samples and standards are treated in exactly the same manner. This includes specimen preparation and specimen presentation to the instrument.

7.4.4 CALCULATIONS AND REPORTING

In the event that the free-pressed-pellet sample method is used (i.e., pressed without binder), the spectrometer results represent final sample concentrations, and no further calculations are required. Similarly, both loose powders (i.e., pure, fine materials) and undiluted liquids measured in disposable sample containers require no further calculation and the spectrometer results represent final sample concentrations. Any material that has been diluted, such as a liquid or pressed pellet, will require additional calculation. Most modern XRF instrumentation comes with software packages that include calculation functions that accommodate dilution factors and automatically back-calculate sample concentrations. For diluted, pressed-pellet samples, the reported units may be weight % or ppm. The actual units for the concentrations are of little importance, as long as care has been taken to work with the proper conversion factors. If a calibration for liquid samples is setup to deliver results in µg/mL, the final concentration of a given element can be calculated in µg/g from the solution element concentration in µg/mL.

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⟨1736⟩ APPLICATIONS OF MASS SPECTROMETRY

1. INTRODUCTION

Mass spectrometry (MS) is an integral part of modern pharmaceutical research and development in academic, industrial, and clinical laboratories. Trace analytical measurement (both qualitative and quantitative), specifically the demand for trace-mixture analysis, has increased the need for this powerful tool. In many cases, the analytical demands of trace-mixture sample analysis has made MS the method of choice for qualitative and quantitative assays for target analytes such as proteins, peptides, drug substances, metabolites, impurities, and degradation products. Because of its analytical capabilities, MS has found widespread application in the pharmaceutical industry. Specifically within the compendial context, MS has been, or has the potential to be, applied to both qualitative (identification tests) and quantitative measurements (assays).

For qualitative tests, MS-based methods can provide molecular mass information via the detection of the molecular ion or ions related to the molecular mass of the analyte as a first level or step of identification. A variety of instrumental methods permit molecular mass determinations for a wide range of materials, up to and including large biomolecules (e.g., proteins) and polymers. Along with the molecular mass information, MS also can provide unique structural information via the generation of fragment ions. The diversity of fragmentation approaches available in modern MS allows structurally significant fragmentation approaches for a wide variety of compendially relevant materials (e.g., small molecules and peptides).

Molecular mass thus can become a surrogate for confirmation or can even be used for the identification of a targeted compound, particularly when used in conjunction with an authentic standard or a chromatographic method. Advanced studies that involve one or two more dimensions of mass analysis also can be used to obtain specific structural detail (fragment ions that correspond to structurally unique portions of the target molecule) or more selectivity to enable powerful approaches for quantitation. Moreover, higher resolution methods that feature mass spectrometry and chromatography can routinely provide a benefit to the scientific community.

Modern analytical MS includes a diverse range of available instrumentation and experimental approaches. The specific mass spectrometer, and, of course, specific chemistries (i.e., sample preparation, chromatography, and ionization) define the final analytical procedure.

2. MASS SPECTROMETERS

The following sections describe the principles of MS, including the general layout of modern MS instrumentation used in the pharmaceutical industry and the specific components (sample introduction, ionization, and mass analyzer). This section also includes a brief description of the operational modes of a mass spectrometer, focusing on fragmentation production and analysis.

2.1 Overview

Analytical measurements that use MS generally involve the following processes: sample preparation, chromatography or sample introduction, ionization, and mass analysis and detection. The resulting output from the mass spectrometer is depicted

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in a mass spectrum, a survey of ions made in the ion source, and is represented in a graphic representation of mass-to-charge ratio (m/z) versus intensity.

Ions are separated by a property of mass such as m/z, where m is the mass of the ion and z is the number of charges on the ion. The method by which ions are separated typically defines the mass spectrometer type. Regardless of type, the mass analyzer separates ions according to the m/z. The mass analyzer continuously acquires data across a predefined range of masses to generate the resulting mass spectra.

2.2 General Layout

A general layout of a mass spectrometer platform for pharmaceutical analysis is shown in *Figure 1*. Samples are prepared via a procedure defined for the specific sample and analyte. The resulting sample then is injected into the mass spectrometer via a chromatographic system such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) or via direct injection. From the inlet, the discrete analytes are ionized for subsequent analysis by the mass spectrometer. All mass spectrometers require four components:

1. Sample introduction technique
2. An ionization source to charge the analyte
3. A mass analyzer to separate the analytes on a mass/charge (m/z) scale
4. A detector to measure the ions.

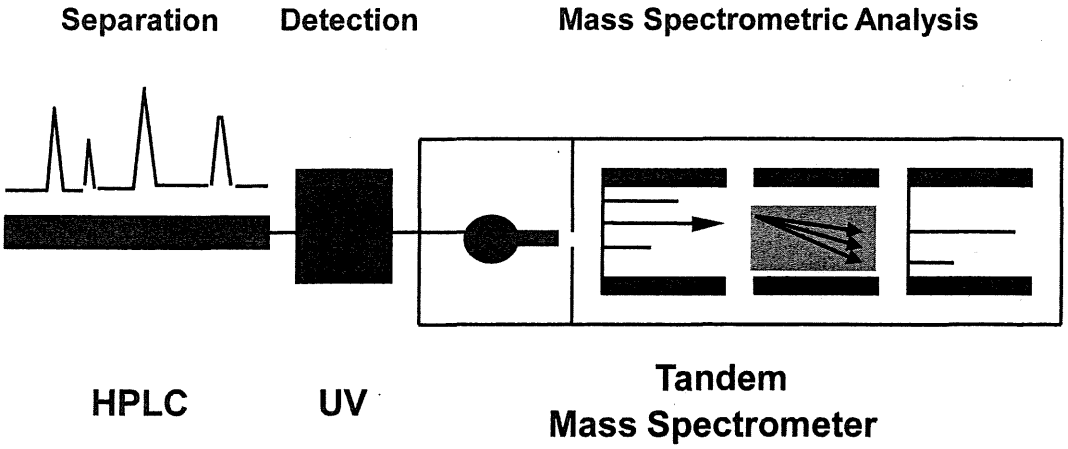

Figure 1. General MS system. HPLC = high-performance liquid chromatography; UV = ultraviolet detector. (Courtesy of Milestone Development Services, Newtown, PA)

MS can be categorized by the dimensions of mass analysis: single-stage mass spectrometers, hybrid or tandem mass spectrometers (MS/MS), and multiple stages of mass analysis (MS^n). These mass spectrometer formats are described in this section.

2.2.1 SINGLE-STAGE MS

A single-stage mass spectrometer provides one dimension of mass analysis. As shown in the schematic in *Figure 2*, a single mass spectrometer can be viewed simply as providing a survey of all ions generated in the ion source. For example, if a 10-component mixture is injected into a chromatograph and each component is separated with adequate resolution, then the mass spectrometer would provide discrete measurement of mass-to-charge of each of the 10 components. The resulting mass spectrum of each component is generated for subsequent interpretation and assignment of molecular mass.

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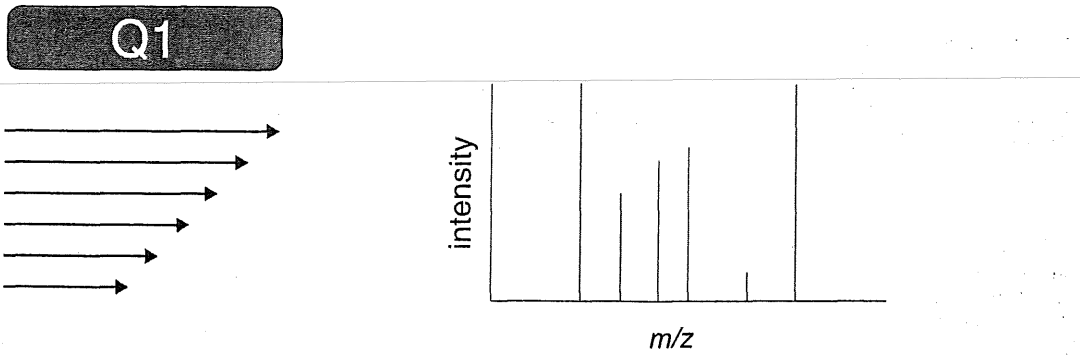

Figure 2. One-dimensional MS scan. Q1 = single-stage mass spectrometer or a single stage of analysis. (Courtesy of Milestone Development Services, Newtown, PA)

2.2.2 TANDEM MASS SPECTROMETRY (MS/MS)

A system capable of carrying out two sequential *m/z* analysis events (i.e., a tandem mass spectrometer) provides increased selectivity for detailed structure elucidation or quantitative analysis.

2.2.3 MULTIPLE STAGES (MS^n)

A unique feature of some MS formats is the capability to perform multiple stages of MS and to generate further fragment information. These mass spectrometers are capable of isolating an ion of interest, inducing fragmentation, isolating a specific product ion and then repeating this process on the resulting or selected product ion.

2.3 Sample Introduction

Several approaches can be used to introduce the sample into the mass spectrometer. The direct, infusion, and chromatographic procedures are described in this section.

2.3.1 DIRECT INTRODUCTION

For some sample types and applications, a direct insertion probe is used to introduce the sample into the mass spectrometer. The sample, usually a pure compound or relatively pure compound, is dissolved in an appropriate solvent. A small amount of the sample (typically 1 µL or less) is deposited onto the probe. Typically, the probe consists of a metal filament, such as platinum, located at its tip. The metal filament is heated, and the sample is desorbed into the ion source of the mass spectrometer. The probe then is removed from the mass spectrometer and is prepared for the next sample.

2.3.2 INFUSION INTRODUCTION

Sample introduction via infusion typically is done to provide a relatively long analysis time or perhaps to conduct a quick survey of a sample. Infusion introduction also can be done when analysts optimize the instrumental conditions (source and mass spectrometer operational parameters) for a specific analyte as well as to obtain greater numbers of spectra. This sample introduction method may require more sample than conventional flow rates. However, infusion sample introduction is widely practiced for nanospray ionization applications for the analysis of proteins and peptides as well as during specialized nanospray applications with small molecules in drug metabolism to determine structure or equimolar response ratios.

2.3.3 CHROMATOGRAPHIC INTRODUCTION

The use of chromatography methods for sample introduction into the mass spectrometer is perhaps the most common approach.

2.3.3.1 Gas chromatography: GC procedures are preferred for nonpolar, volatile analytes (see *Chromatography* ⟨621⟩. Samples contained in solvent are injected onto the injection port of the GC. The sample is volatilized, and a nonreactive, inert gas such as helium carries the sample through the GC column, which is contained in a temperature-controlled oven. The combination of carrier gas and heat moves the analyte through the column. Capillary GC columns are commonly used in pharmaceutical applications. Molecules separate within the capillary column and are introduced directly into the ion source of the mass spectrometer.

2.3.3.2 High-performance liquid chromatography: A popular approach for sample introduction into the mass spectrometer involves HPLC (see ⟨621⟩). HPLC procedures are preferred for nonvolatile and thermally labile analytes, but are suitable for use with any analyte that is readily ionizable in a solution environment with the appropriate chemical modifiers. Samples are prepared in solution and then injected onto the HPLC column. Analytes are separated based on the partitioning between the mobile phase and stationary phase and are introduced into the ion source of the mass spectrometer. The effluent is volatilized in the ion source and ionization occurs. The subsequent ions then are introduced into the mass spectrometer for analysis.

2.3.3.3 Capillary electrophoresis: Capillary electrophoresis (CE), also called capillary zone electrophoresis (CZE), is a separation method that exploits subtle differences in the ionic composition of analytes to separate them based on electrophoretic mobility

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in a conductive liquid. Details regarding the principles and use of this methodology can be found in *Capillary Electrophoresis* ⟨1053⟩. During the past 15 years this separation method periodically has been used with MS as an alternative to LC–MS for certain classes of compounds. However, with the widespread use of HPLC in analytical and MS laboratories, CE-coupled MS methods often are superseded by LC–MS methods.

2.4 Ion Polarity

Ionization involves the process of converting the analyte into the gas phase and depositing a charge onto the molecule. The final charge of the analyte determines the ion polarity to be used in the mass analysis. Generally, selection of the ionization mode depends on the ability of the desired analyte to accept or lose a proton during the ionization process. MS can be performed in the positive or negative ion polarity mode.

2.4.1 POSITIVE ION MODE

Many MS pharmaceutical analyses use the positive ion mode. For example, compounds that contain a basic functional group such as an amine (e.g., proteins and peptides) are excellent candidates for MS analyses in the positive ion mode because under acidic conditions these compounds readily accept a proton to form positively charged ions.

2.4.2 NEGATIVE ION MODE

The negative ion mode is well suited for compounds that can easily lose a proton. Compounds that contain a carboxylic acid, phosphate, or a sugar, for example, are good candidates for negative ion MS.

2.5 Ionization Procedures

A variety of ionization procedures can be used with a mass spectrometer for pharmaceutical analysis. The various ionization procedures are described in this section.

2.5.1 ELECTRON IONIZATION

Electron ionization (EI) is used mostly with GC applications when the analytes of interest are nonpolar and are easily volatilized. Mass spectra produced by EI procedures are characterized by extensive fragmentation. Because EI typically produces extensive fragmentation, EI is considered a hard-ionization mode. Mass spectra are obtained when a 70 eV electron beam enters the source and impacts the analyte molecules present in the gas phase:

$$M + e^- \rightarrow M^{+\cdot} + 2e^-$$

Nearly all EI applications are conducted in the positive ion mode. These spectra are highly reproducible. Thus, EI spectral libraries can be used to determine the structure and to confirm the identity of unknown compounds. Additionally, fragmentation patterns have been extensively studied for electron ionization and can help determine structure of unknown compounds.

2.5.2 CHEMICAL IONIZATION

Chemical ionization (CI) procedures rely on electron ionization of reagent ions such as methane, ammonia, or isobutene. The reagent ions react with the analyte molecules (ion–molecule reaction) in the source of the mass spectrometer. In the positive ion mode, analyte molecules are ionized via proton transfer and/or adduct formation, producing even electron molecular ion species or adduct ions. Depending on the analyte, both positive and negative ion spectra can be obtained with CI procedures. CI is considered a softer ionization (less fragmentation of the molecular ions) mode than EI although some approaches can induce fragmentation depending on the CI gas chosen and the structure of the analyte molecule. CI is very useful for reactive and unstable compounds where a molecular mass determination is desired. Representative CI ionization reactions for both GC–MS and LC–MS methods follow.

Protonated molecule formation (GC–MS example):

$$CH_4 + e^- \rightarrow CH_4^+ + 2e^-$$
$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3$$
$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$
$$M + C_2H_5^+ \rightarrow [M + H]^+ + C_2H_4$$

Protonated molecule formation (LC–MS example):

$$M + NH_4^+ \rightarrow NH_3 + [M + H]^+$$

Adduct ion formation (LC–MS example):

$$M + NH_4^+ \rightarrow [M + NH_4]^+$$

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2.5.3 ATMOSPHERIC PRESSURE IONIZATION

Atmospheric pressure ionization (API) procedures allow the direct introduction of samples into the mass spectrometer from a liquid interface such as an HPLC. The two most common forms of API used in pharmaceutical analyses are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

2.5.3.1 Electrospray ionization: ESI is a method that produces fine charged droplets of a liquid phase that carries the analyte of interest. The liquid phase typically is a volatile combination of water and organic solvent (e.g., acetonitrile or methanol). A small percentage of a reagent (e.g., 0.1% formic acid) also is included to increase the conductivity of the solution. ESI typically is used with HPLC and involves the nebulization of the sample delivered at flow rates that range from nL/min to mL/min to produce a fine spray of droplets (radius = 0.5–1.0 µm). Solvent evaporation results in an increased charge concentration at the droplet surface until ions are liberated directly from the droplet. Ions are transported or focused directly into the mass spectrometer, and the resulting spectra contain ions that are typically indicative of the molecular mass of the analyte.

ESI often is referred to as a soft-ionization procedure because typically it does not result in fragmentation of the analyte during the ionization process. The development of this ionization technology was crucial for the analysis of large biopolymers (e.g., proteins) because it allows the addition of multiple charges to a single molecule, thus bringing the *m/z* of the analyte into a suitable *m/z* range for many types of mass analyzers. For example, *Figure 3* shows the ESI charge state distribution for equine heart apomyoglobin as analyzed on a simple single quadrupole instrument with an upper scan limit of *m/z* 2000. The series of multiply charged ions allows the spectrum to be collected within the instrument's working *m/z* range. The ability to calculate the intact mass of the protein from a combination of all the charge states allows a more accurate average mass (see inset in *Figure 3*) to be determined (within 0.1% on a simple quadrupole instrument). Thus ESI methods are critically important for the analysis of large biomolecules.

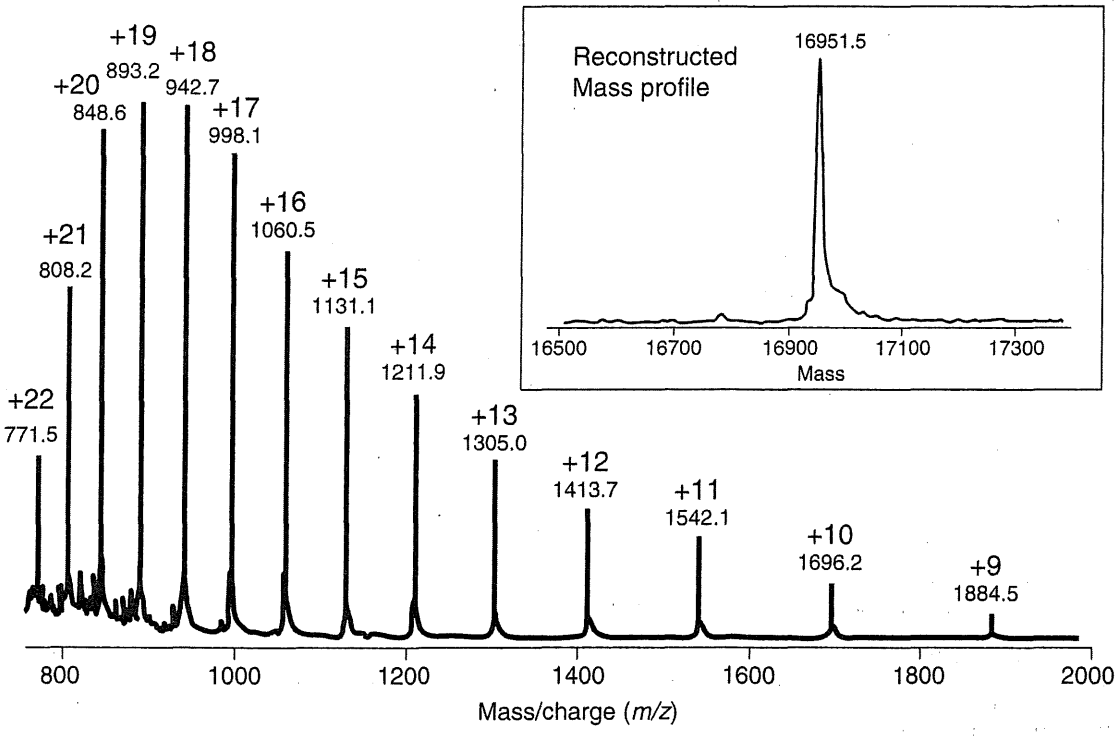

Figure 3. Electrospray ionization mass spectrum for equine heart apomyoglobin.

2.5.3.2 Atmospheric pressure chemical ionization: APCI procedures are used with HPLC and involve the nebulization and heating of the sample to liberate neutral molecules. A corona discharge produces reagent ions (e.g., H_3O^+ or NH_4^+) from the mobile phase. The reagent ions react with analyte molecules via proton transfer or adduct formation. The resulting positive ion spectrum typically contains $[M + H]^+$ ions that are indicative of molecular mass and fragment ions that correspond to unique substructure(s) of the analyte molecule. Negative ion spectra are generated when reagent ions (e.g., OH^- or CH_3COO^-) react with the analyte molecule to produce $[M - H]^-$ ions.

2.5.4 MATRIX-ASSISTED LASER DESORPTION IONIZATION

Matrix-assisted laser desorption ionization (MALDI) is a soft-ionization procedure used primarily for biomolecules that relies on the addition of a chemical matrix dried with the analyte of interest. This matrix compound can absorb laser energy at a particular wavelength during the laser ablation process. By a mechanism that has not been fully elucidated, some of the ions generated in the matrix can transfer protons to the analyte, and the resulting gas-phase ions are focused into the mass spectrometer. Specific matrices have been empirically developed for various classes of analytes, including 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid for proteins and peptides and picolinic acid for

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oligonucleotides. Unlike ESI, MALDI typically produces lower charge-state ions, with singly charged ions being the most favorable.

2.5.5 AMBIENT IONIZATION PROCEDURES

Ambient ionization generally refers to a collection of MS procedures that permit direct sampling and interrogation of analytes from sample matrices or surfaces under ambient conditions with little or no pretreatment. In recent years, the number and type of ambient ionization procedures have rapidly expanded. This is evident from the proliferation in recent literature of acronyms that represent various ambient ionization procedures. Ambient ionization has enjoyed broad application in fields that include forensics; food safety; monitoring of environmental contaminants; polymers; fuels; detection of explosives and drugs of abuse; molecular imaging of surfaces and tissues; profiling and characterization of metabolites, proteins, and biomolecules; as well as monitoring of chemical reactions and processes.

Ambient ionization procedures typically produce analyte ions directly from sample surfaces or require initial production of analytes that subsequently are ionized by any of several processes. Therefore, ambient ionization procedures can be broadly categorized as representing either direct or multistage ionization mechanisms. Direct ionization procedures generate analyte ions from a sample solution or droplet in an electric field, or desorb analyte ions by impinging a sample surface with charged droplets or solvent ions, photons, or metastable atoms. Multistage ionization procedures initially produce analyte particle or droplets with a liquid or gas stream, thermal desorption, irradiation or ablation with a laser, or nebulization. Analytes subsequently react with a charged species or with metastable atoms generated by ESI, APCI, or photoionization to produce analyte ions.

Table 1 is a representative, but not comprehensive, listing of ambient ionization procedures and illustrates the diversity and similarity among various approaches.

Table 1. Ambient Ionization Procedures

Acronym	Description	Mechanisms
APGDDI	Atmospheric pressure glow discharge desorption ionization	Thermal desorption, gas discharge ionization
APPeI[a]	Atmospheric pressure Penning ionization	Similar to APCI
AP–TD/ESI[a]	Atmospheric pressure thermal desorption/electrospray ionization	Thermal desorption, ESI
ASAP[a]	Atmospheric pressure solids analysis probe	Thermal desorption
DAPCI	Desorption atmospheric pressure chemical ionization	Thermal desorption, APCI
DAPPI[a]	Desorption atmospheric pressure photoionization	Thermal desorption, photoionization
DART[a]	Direct analysis in real time	Gas discharge ionization
DBDI	Dielectric barrier discharge ionization	Thermal desorption, gas discharge ionization
DCBI	Desorption corona beam ionization	Thermal desorption, APCI
DESI	Desorption electrospray ionization	Similar to ESI
EADESI	Electrode-assisted desorption electrospray ionization	Similar to ESI
EASI	Easy ambient sonic spray ionization	Supersonic spray ionization
EESI[a]	Extractive electrospray ionization	Similar to ESI
ELDI[a]	Electrospray laser desorption ionization	Laser desorption or ablation, ESI
FD–ESI[a]	Fused droplet electrospray ionization	Similar to ESI
IR–LADESI[a]	Infrared laser-assisted desorption electrospray ionization	Laser desorption or ablation, ESI
LAESI[a]	Laser ablation electrospray ionization	Laser desorption or ablation, ESI
LD–APCI[a]	Laser desorption atmospheric pressure chemical ionization	Laser desorption, APCI
LD–ESI[a]	Laser desorption electrospray ionization	Laser desorption, ESI
LDSPI[a]	Laser desorption spray postionization	Laser desorption or ablation
LDTD[a]	Laser diode thermal desorption	Thermal desorption
LEMS[a]	Laser electrospray mass spectrometry	Laser desorption or ablation

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Table 1. Ambient Ionization Procedures *(continued)*

Acronym	Description	Mechanisms
LESA[a]	Liquid extraction surface analysis	Similar to ESI, surface extraction with solvent droplet
LIAD–ESI[a]	Laser-induced acoustic desorption electrospray ionization	Laser desorption or ablation, similar to ESI
LPI–MS[a]	Liquid surface Penning ionization mass spectrometry	Similar to APCI
LSI	Laser spray ionization	Laser desorption or ablation
MALDESI[a]	Matrix-assisted laser desorption electrospray ionization	Laser desorption or ablation
PADI	Plasma-assisted desorption ionization	Thermal desorption, gas discharge ionization
SESI[a]	Secondary electrospray ionization	Similar to ESI
TDAMS[a]	Thermal desorption based ambient mass spectrometry	Thermal desorption
TD/APCI[a]	Thermal desorption/ atmospheric pressure chemical ionization	Thermal desorption, similar to APCI

[a] Multistage ionization procedure.

3. MASS ANALYSIS

The method by which ions are separated typically defines the mass spectrometer type. Essentially, the mass analyzer is responsible for filtering the ions generated during the ionization process. The various mass analyzers that typically are used in the pharmaceutical industry are described in this section.

3.1 Quadrupole

A quadrupole mass spectrometer consists of a set of four parallel rods. When a combination of constant (DC) and alternating (AC) voltage are applied to the opposing rods respectively, the resulting electric fields allow ions of a specific m/z to stably transit the quadruple and to pass through to the detector. Quadrupole mass spectrometers are relatively low-cost instruments and provide good qualitative and quantitative analytical capabilities. Generally, quadrupole mass spectrometers are limited to production of low-resolution mass spectra.

3.2 Magnetic Sector

A magnetic sector mass spectrometer filters ions by the means of the application of a magnetic field. The magnetic field is varied, and ions are deflected to follow a curved path so that ions with different m/z ratios are separated.

3.3 Ion Traps and Ion Cyclotron Resonance

Most commercial mass spectrometers fall broadly into two categories: those that rely on continuous beams of ions being sent to a detector for sequential m/z detection (like cars on a highway driving from point A to point B), and those devices that "trap" ions in discrete, repeating orbits (like cars on a race track). The latter devices are broadly referred to as *ion traps*. The orbital paths are complex and unique to each device. The ions can be trapped by static electric fields (DC voltage), dynamic quadrupole electric fields [radio frequency (RF) AC voltage], magnetic fields, or some combination of the types. Detection of the ions may take place by sequential ion ejection (after trapping, by m/z) or by detection of all ions simultaneously [while trapped, i.e., by image current detection and subsequent Fourier transformation (FT) of the data]. Examples include the popular linear and 3D ion trap MS systems (RF AC voltage only with ion ejection for detection), the FT Orbital Trapping MS (which uses DC voltage and a quadrupole field for trapping, with image current detection), and the less common FT Ion Cyclotron Resonance MS (which uses a superconducting magnet to provide the primary trapping field). The FT devices generally provide very high mass resolution (e.g., 10^5–10^6 at m/z 400) at reasonable scan speeds (1 Hz), and the RF-based devices generally offer fast scan rates (0.1 Hz) but lower resolution (10^3). Lastly, the most popular FT equipment is now hybridized with a quadrupole RF device (especially the linear trap) to yield instrumentation with multiplexed experiment capabilities (multiple sequential MS–MS experiments with accurate m/z determinations for structure elucidation).

3.3.1 TIME-OF-FLIGHT

A time-of-flight (TOF) mass spectrometer uses differences in transit time through a field-free drift region to separate ions. Ions generated in the ion source are pulsed into the field-free drift region (flight tube) by an electric field. Lighter ions have a higher velocity and reach the detector sooner. The TOF mass spectrometer has benefited significantly from the use of fast electronics and fast computers to provide systems characterized by high speed, high sensitivity, and high resolution.

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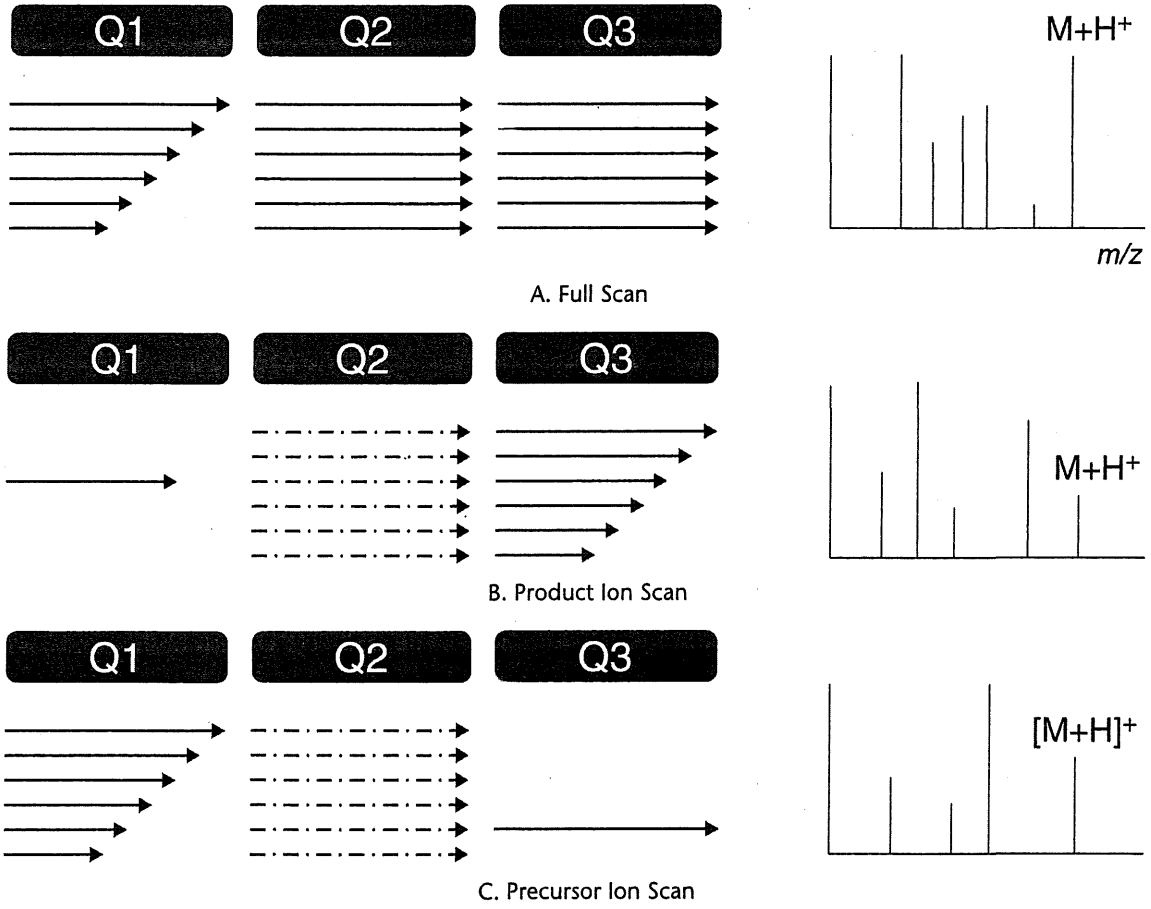
4. MS/MS AND MS^N SPECTROMETRY INSTRUMENTATION

A wide variety of tandem MS instruments are available to the pharmaceutical research and development community. Each of the tandem instruments has some unique features that make it particularly suited for some specific applications, but this does not preclude the use of other tandem instruments to meet a specific analytical need. The appropriate selection of a tandem instrument that is suitable to meet a specific need depends on the type of analysis required (e.g., quantitation or structural elucidation), a variety of sample-specific factors (e.g., analyte concentration or the complexity of the matrix), and whether sample fractionation is incorporated before the introduction in the mass spectrometer (e.g., by LC–MS). A brief summary of some of the common types of tandem instruments follows, highlighting some of the typical applications.

4.1 MS/MS and MS^n Spectrometers

4.1.1 TRIPLE QUADRUPOLE

The triple quadrupole mass spectrometer is used for both qualitative and quantitative analysis. A triple quadrupole mass spectrometer features a first stage of mass analysis (Q1) for the selection of a precursor ion, followed by an RF-only collision quadrupole region (Q2). A second stage of mass analysis (Q3) is used for product ion analysis. Most quantitative LC–MS-based analyses feature the use of triple quadrupole mass spectrometers. Furthermore, the triple quadrupole mass spectrometer is capable of providing all the most common qualitative scan modes: full scan, product ion, precursor ion, and neutral loss (see *Figure 4* and *4.2 MS/MS and MS^{n}Spectrometry Operational Modes*).



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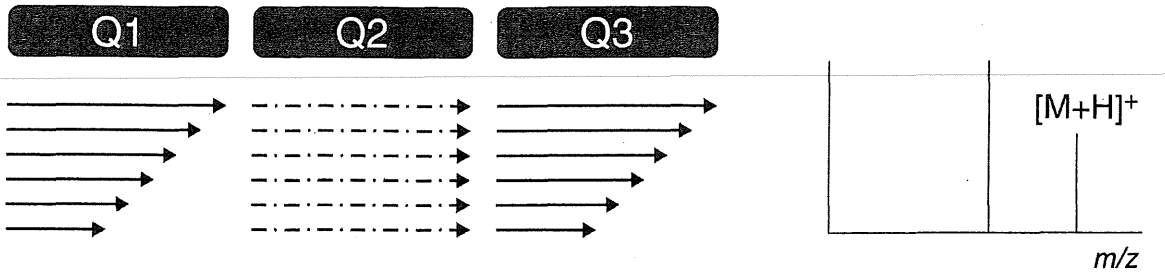

D. Neutral Loss Scan Figure 4. Triple quadrupole MS. (A) *Full scan*—Q1 scans the ions produced in the ion source. This scan also can be conducted with Q3. (B) *Product ion scan*—Q1 is set to select a specific molecular ion produced in the ion source. Fragmentation of the ion(s) selected in Q1 occurs in the collision cell (Q2). Q3 scans the resulting fragments. The product ion spectrum contains all the fragments of the selected precursor ion(s). (C) *Precursor ion scan*—Q1 scans the ions produced in the ion source. Ions fragment in the collision cell (Q2). Q3 is set to select a specific product ion (derived from the product ion spectrum). The scan results in a spectrum that contains all precursor ions that generate the specific product ion. (D) *Neutral loss scan*—Q1 scans the ions produced in the ion source, and ions fragment in the collision cell (Q2). Q3 also scans, but at a difference in mass equal to a selected neutral loss (as derived from the product ion spectrum). The scan results in a spectrum that contains all molecular ions that generate a specific neutral loss fragment. (Courtesy of Milestone Development Services, Newtown, PA)

4.1.2 TOF–TOF SYSTEM

A tandem configuration for TOF systems typically is used for the characterization and sequencing of peptides and other biomolecules from fairly simple mixtures (e.g., proteolytic digestion of a single-protein therapeutic). These systems often are configured for ionization by MALDI, which allows peptide sequence confirmation without the need for an initial separation. The relative ease of use of such instruments and the robust fragmentation data collected for confirmation of peptides may allow TOF–TOF instruments to be used for monograph identification testing in the future.

4.1.3 MAGNETIC SECTOR

Tandem configurations for magnetic sector instruments traditionally have been used for analyses that require high resolution and mass accuracy, such as structural elucidation of unknowns. However, because of greater resolution and mass accuracy becoming available in other tandem MS platforms (e.g., ion traps and quad-TOF devices), magnetic sector instruments are now less commonly used for these purposes.

4.1.4 ION TRAP

The tandem configuration for ion trap systems allows the isolation and fragmentation of ions in a time-dependent manner, unlike other tandem instruments that have spatially distinct mass analyzers in the path of the ion beam. One advantage of the trap-isolate-fragment-detect mechanism in an ion-trap instrument is the ability to re-isolate product ions from the initial precursor ions for further fragmentation. This sequential fragmentation allows more detailed structural determination to assist in elucidating structural unknowns. For protein and peptide applications, ion-trap instruments have been widely used to identify and sequence peptides and to characterize protein modifications (e.g., phosphorylation and glycosylation).

4.1.5 FOURIER-TRANSFORM ION CYCLOTRON RESONANCE

Fourier-transform ion cyclotron resonance (FT–ICR) mass spectrometers, like ion traps, facilitate a time-dependent isolation of ions. FT–ICR mass analyzers offer high resolution (typically 10^5–10^6) and can achieve sub-ppm mass accuracy, which permits determination of accurate mass and elemental composition for unknown compounds with a high degree of certainty. Ions can be selectively excited or ejected from the ICR cell by generation of a waveform that contains resonant frequencies of the *m/z* values of the ions of interest. Several fragmentation procedures are available for achieving the structural characterization of small and large molecules with FT–ICR MS, including collision-induced dissociation (CID), electron capture dissociation (ECD), and infrared multiphoton dissociation (IRMPD). Some commercial FT–ICR MS systems use a tandem ion trap to isolate and fragment precursor ions, the product ions of which are subsequently transferred to the ICR cell for detection or additional fragmentation by other procedures. The high resolution and mass accuracy of FT–ICR MS make this instrument particularly well suited for the structural characterization of components that comprise highly complex mixtures of small and large organic molecules. FT–ICR MS also has been widely used in the characterization of lipids and carbohydrates and in the characterization and sequencing of peptides and proteins, including examination of unique features such as post-translational modifications.

4.1.6 HYBRIDS

A variety of hybrid tandem mass spectrometers have emerged to either enhance specific applications or to broaden the overall utility of the instrument in a variety of applications. Currently the most common hybrid instrument used for pharmaceutical characterization is the quadrupole TOF (Quad-TOF) MS, which combines the scanning speed of a quadrupole mass filter with the resolving power and mass accuracy of the TOF mass analyzer. This combination is well suited for structural elucidation because the improved resolution and mass accuracy of the TOF (by comparison with a quadrupole) allow a greater degree of certainty in determining the probable elemental makeup of both the precursor and product ions. In addition, the

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use of a quadrupole mass filter in this tandem configuration allows scanning functions like those outlined for a triple-quadrupole instrument (*Figure 4*), although additional postprocessing of the data is required. An increasing number of hybrid configurations have been marketed to take advantage of the unique feature of the combinations for resolution, mass accuracy, and quantitation. Some of these configurations include Quadrupole–Trap, Trap–TOF, Trap–IRC, and orbital trapping mass spectrometers.

4.2 MS/MS and MS^n Spectrometry Operational Modes

4.2.1 FRAGMENTATION PRODUCTION MODES

Collision-Induced Dissociation (CID) or Collisionally Activated Dissociation (CAD) is the process by which a selected precursor ion undergoes collisions with neutral gas molecules in a collision region to yield product ions. Fragmentation occurs at relatively low energies (1–100 eV) primarily at the site(s) of ionization to yield a fragment ion and a neutral molecule. Nitrogen, argon, or helium typically is used as the collision gas with collision energies in the 10–50 eV range.

Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD) are two distinct but related fragmentation methods used primarily to fragment proteins and peptides to generate *c*- and *z*-type ions that are useful for determining protein sequence and characterization of protein modifications. The fragmentation in each case relies on the introduction of either a free electron (ECD) or a radical anion (ETD) into the gas phase of positively charged molecules to induce chemical fragmentation. The advantage of these methods for proteins and peptides versus CID is that the chemical fragmentation occurs almost exclusively along the peptide backbone, thereby preserving the position and context of protein modification that often are lost during CID.

4.2.2 fragmentation analysis modes

The most common MS/MS scan modes are described in this section.

4.2.2.1 Product ion: The product ion scan mode is illustrated in *Figure 4B* for a triple-quadropole (QQQ) instrument. Application of the product ion scan mode is performed when the first mass analyzer (Q1) is held at a specific *m/z*. This ion, typically the protonated molecule, $[M + H]^+$, is fragmented in the collision cell (Q2). The second mass analyzer (Q3) scans the resulting product ions. For a trapping device such as an ion trap mass spectrometer, ions of a specific *m/z* are isolated and are collisionally activated, followed by scanning of the product ions. In either case, the resulting product ions' MS–MS spectrum contains the diagnostic fragment ions indicative of the characteristic substructures of the selected analyte. This scan mode requires that a full scan mass spectrum be obtained before the product ion experiment to determine the appropriate selection of the molecular ion (typically $[M + H]^+$). The product ion scan mode is available in all mass spectrometers capable of MS–MS operation.

4.2.2.2 Precursor ion: The precursor ion scan mode is illustrated in *Figure 4C*. Application of the precursor ion scan mode is performed when the first mass analyzer (Q1) is scanned and allows all ions created in the ion source to pass into the collision cell (Q2). The second mass analyzer (Q3) is held at a specific *m/z* ratio that corresponds to a diagnostic or unique fragment ion of the molecule or class of molecules. The resulting precursor ion MS–MS spectrum contains all molecular ions that contain a diagnostic fragment ion. The precursor ion scan mode requires that the product ion experiment be performed to confirm the diagnostic fragment ion(s). This scan mode can be used to screen for molecules that contain a specific substructural feature such as a peptide residue. The precursor ion scan mode is available on triple-quadrupole, magnetic sector, and some hybrid instrumentation.

4.2.2.3 Neutral loss: The neutral loss scan mode is illustrated in *Figure 4D*. Application of the neutral loss scan mode is performed when both mass analyzers (Q1 and Q3) are scanned at the same rate. The second mass analyzer (Q3) is offset (lower) from the first by a constant *m/z* difference. Molecular ions enter Q1 and fragment in the collision cell (Q2), and the resulting fragment ions are detected after passage through Q3. Because of the offset scan function described above, the resulting spectrum presents all molecular ions that have undergone the selected neutral loss. This scan mode also requires that the product ion scan be performed to confirm the diagnostic neutral loss(es). Like the precursor ion scan mode, this scan mode is used to screen for molecules with a diagnostic structural feature such as phosphate or glucuronide conjugation. The neutral loss ion scan mode is available on triple-quadrupole, magnetic sector, and some hybrid instrumentation.

4.2.2.4 MS^n: The MS^n acquisition mode is essentially a sequential application of the product ion scan discussed in *4.2.2.1 Product ion*. That is, it is used to further fragment product ions from a previous product ion experiment. Thus it can be utilized to obtain additional structural information for identification of analytes. This acquisition mode typically is available only on ion trap or FT–ICR instruments.

5. QUALITATIVE ANALYSIS

MS is a powerful method and important tool for structure identification because of its ability to provide information about the mass, elemental composition, and structural features of known and unknown molecular entities. In its simplest terms, an MS separates and mass-measures ions related to the sample. A mass spectrometer separates, detects, and records the presence of ions, not neutral molecules. For the purposes of small-molecule organic MS, the charge state (*z*) typically is 1.

A wide range of mass spectrometers and ionization interfaces are available for specific types of MS measurements. Thus a diverse range of experimental approaches can be employed for any given MS measurement needed. Some of the more common and applicable approaches are presented in this section. The appropriateness of a given MS experimental approach is demonstrated by the respective validation data provided in the submission supporting a new monograph or monograph procedure.

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5.1 The Mass Spectrum

The mass spectrum typically is displayed as a plot of *m/z* on the abscissa versus ion intensity as the ordinate and frequently is normalized to the most intense ion in the spectrum. Some characteristic features in a typical full-scan mass spectrum include (depending on ionization procedure) the molecular ion (e.g., M^+), protonated or deprotonated molecules ($[M + H]^+$ or $[M - H]^-$), or adduct ions (e.g., $[M + NH_4]^+$), all of which are indicative of the molecular mass of the analyte. Mass spectra may be more or less complex, depending on the specific analyte(s) and ions detected. EI mass spectra typically are rich in fragment ions, but API and soft-ionization procedures may produce spectra that are sparse in fragment ions but often feature more prominent or base peak ions that are indicative of MW. Therefore, the interpretation of mass spectra may be more or less straightforward. Typically, the availability of any supporting information about the sample (e.g., origin, history, preparation, stability, solubility) can greatly assist with interpretation. Two primary considerations merit initial discussion because of their importance in the interpretation of mass spectra: resolution and mass accuracy.

5.1.1 MASS RESOLUTION

In MS, resolution is the extent to which two ions of adjacent *m/z* can be distinguished from each other, and has been expressed in different ways. Two adjacent peaks in a spectrum at values *m* and $m - \Delta m$ may be separated by a valley, which at its lowest point is a specified maximum amount of overlap between the peaks. The resolution would be expressed as $m/\Delta m$. For example, the term "10% valley" describes a 10% amount of overlap between two peaks and is appropriate when the peaks are of equal height and shape. In practice, however, adjacent peaks of equal height and shape are rarely observed. Therefore, resolution also can be calculated by using the apparent width of a single peak at a given point in mass range as Δm, and it often is expressed as the measured width of a peak at half-height (full-width half-height maximum, or FWHM). Resolution is an important factor in establishing mass accuracy, evaluating isotope patterns and abundance, determining charge state of multiply charged ions, and distinguishing nominally isobaric interferences.

Nominal mass spectrometers instruments typically provide mass resolution on the order of 1000, whereas for high-resolution mass spectrometry (HRMS) this can range from 5000 to 1,000,000. Improved resolution includes the ability to separate and distinguish isobaric interferences (analytes with the same nominal mass but with different empirical formulae) and to mine complex data sets more efficiently (using increased specificity).

5.1.1.1 Nominal resolution: A number of different types of mass spectrometers are capable of generating nominal resolution mass spectra. Probably the most common mass spectrometer employed for nominal mass resolution work is the quadrupole mass spectrometer. In a quadrupole mass spectrometer, ions pass through a set of four rods such that opposing rods ramp through RF or DC, effectively scanning through the desired *m/z* range and allowing only a single *m/z* to pass at any given instant. The net effect is to obtain nominal mass resolution (e.g., *m/z* 400 can be reliably distinguished from *m/z* 399 and *m/z* 401) and to obtain a spectrum that can define monoisotopic mass and associated isotopic cluster (e.g., A + 1 and A + 2). With this type of mass spectrometer (nominal resolution), the primary information obtained is an indication of the molecular mass of the analyte and, depending on the experimental approach used (e.g., EI ionization), ion fragments related to the structure of the analyte.

5.1.1.2 High resolution: HRMS has important advantages for the identification of analytes. The various forms of this technology allow determination of the mass of small molecules to the third or fourth decimal place (i.e., approximately 0.1 to 10 ppm mass error). This provides an added degree of specificity (certainty) that, when combined with isotope ratio information and chemical sense, results in elucidation of the analyte's empirical formula as a starting point for further analysis. Thus HRMS offers advantages compared to nominal MS, for which nominal mass is the starting point. HRMS can be performed with a number of different technologies, including TOF–MS, as well as orbital-trapping MS and ion cyclotron resonance MS, where the latter two approaches utilize Fourier transformation to process the raw data. These systems have different advantages, including improved sensitivity for full spectrum data collection (versus scanning instruments like quadrupoles), as well as resolution, mass accuracy, and calculation of the isotopic ratio intensities.

5.1.2 MASS ACCURACY

Mass accuracy is the comparison of an experimentally determined *m/z* to the true *m/z* ratio. Mass accuracy is influenced by a number of factors, including mass resolution, signal abundance, calibration type and algorithms, choice and number of calibrants used, interferences such as chemical noise, and linear dynamic range inherent with a particular type of mass spectrometer. Mass accuracy may be expressed as millimass unit (0.001 μ) differential or ppm. Mass accuracy, as ppm, is given by the following:

$$\Delta m_{acc} = m_{true} - m_{meas}$$

$$ppm = (\Delta m_{acc}/m_{true}) \times 10^6$$

m_{meas} = measured value

m_{true} = calculated or true mass

Although resolution and accuracy may not be directly correlated, HRMS typically allows improved assignment of exact mass. In practice, high resolution and high mass accuracy are both necessary to minimize error introduced into the mass measurement by isobaric interferences. Sufficiently high resolution and mass accuracy together can facilitate assignment of elemental composition to ions in a mass spectrum, assisting with confirmation or elucidation of structure, respectively, for known or unknown compounds.

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5.2 Interpretation of Mass Spectra

When interpreting the mass spectra of unknown compounds, analysts typically can differentiate unknowns into two broad categories.

The first category, representing the situation more commonly encountered, can be described as known unknowns or compounds for which supportive information may be available or can be determined by comparison with standards, related substances, metabolites, synthetic impurities, or intermediates. In this case, the objective is verification of a compound's identity by demonstrating its consistency with a standard material by using predetermined criteria. This objective often is achieved by the direct comparison of mass spectral features of an unknown with either a concurrently analyzed standard or an archived library standard mass spectrum generated with similar instrumentation and conditions. For high-resolution accurate mass spectra, confirmation of identity may include the verification of elemental composition. Characteristic fragmentation patterns obtained under consistent conditions also can be used for the confirmation of identity by providing a fingerprint that indicates functional groups, substituents, and structural differences or modifications. The importance of chromatographic separation (i.e., demonstration of retention characteristics that are similar to a standard) also should be considered as part of an overall identification confirmation. The importance of chromatographic separation is particularly evident with the identification of various structural isomers or enantiomers for which MS alone may not provide sufficient distinction.

The second category comprises true unknowns or compounds for which no or very limited information about structure or background is available and presents the most complex cases for spectral interpretation. True unknowns are encountered less frequently and often necessitate the combination of multiple spectral procedures in order to achieve a complete structural elucidation. For true unknowns, any relevant information (e.g., origin, preparation, matrix, solubility, other compounds if the sample is a mixture) can aid interpretation.

The first step in the interpretation of mass spectra is the determination of monoisotopic mass. This peak is highly dependent on the ionization procedure employed, analytical conditions, and characteristics of the molecules from which the ions are formed. There are prominent differences between EI and API mass spectra, which, for purpose of this discussion, generally result from hard- and soft-ionization procedures, respectively. EI forms primarily an odd-electron radical molecular cation, $M^{+\cdot}$, and API and other soft-ionization procedures typically produce characteristic even-electron protonated $[M + H]^+$ or deprotonated $[M - H]^-$ molecules.

The Nitrogen Rule can be useful in identifying the molecular ion peak: it states that an odd-electron ion containing either no nitrogen or an even number of nitrogen atoms will be observed at even nominal mass and that an odd-electron ion with an odd number of nitrogen atoms will appear at odd nominal mass because nitrogen has an even integer mass (14) and an odd valence (3). This rule holds for all compounds that contain carbon, hydrogen, oxygen, nitrogen, sulfur, and the halogens, as well as phosphorous, boron, silicon, sulfur, and alkaline earth metals. Applied to even-electron ions (e.g., $[M + H]^+$ or $[M - H]^-$), the Nitrogen Rule states that an even-electron ion containing either no nitrogen or an even number of nitrogen atoms will be observed at odd nominal mass and that an even-electron ion with an odd number of nitrogen atoms will appear at even nominal mass.

The formation of molecular adducts can assist with determining and verifying mass in both positive and negative ionization modes. Some common adducts observed in positive ion mass spectra are $[M + Na]^+$, $[M + K]^+$, and $[M + NH_4]^+$ adducts. Adducts with other inorganic or organic cations, such as Li, Ag, Cs, H_2O, acetonitrile, methanol, isopropanol, and small organic protonated or quaternary amines, also can be generated. Adducts present in negative ion mass spectra include those formed with Cl, Br, and formic or triflouoroacetic acid. Multiple adducts are not uncommon in either positive or negative ionization mode mass spectra from API and other soft-ionization procedures and can include combinations of protonated or deprotonated molecules accompanied by additional protons, multiple inorganic or organic cations, or anions. For some applications, it may be desirable to deliberately produce adducts by the modification of the sample solution, matrix, or mobile phase to induce characteristic fragmentation or to denote components in a mixture with common or unique structural features. Dimeric or polymeric analyte ions $[2M + H]^+$ or $[2M - H]^-$ may be observed respectively in positive or negative ion API mass spectra, often with high analyte concentrations. Analogous dimeric adducts with Na, K, and other inorganic cations also can be formed and observed and can provide verification of the MW. Overall, adduct formation and ultimately the presence of adduct ion peaks in mass spectra are highly dependent on ionization procedure, ionization source conditions, type of mass spectrometer or analyzer, sample matrix, mobile phase composition, and the analyte itself.

The relative intensity of mass spectral peaks can provide information about molecular and ion structure. The most intense peak in a mass spectrum is referred to as the base peak and represents 100% relative abundance in normalized mass spectra. In EI mass spectra, the base peak may or may not represent the molecular ion. However, with API and softer ionization procedures, $[M + H]^+$ or $[M - H]^-$ often appear as a prominent peak in the mass spectrum. The relative intensities of peaks in EI mass spectra, and to some extent in API and other mass spectra, can indicate ion stability.

5.3 Elemental Composition and Structure

Each molecule has a specific elemental formula or composition that by itself does not suggest anything about the structure of the molecule, because completely different molecules can have identical empirical composition. The mass spectrum of an analyte indicates its monoisotopic mass, but the larger the mass, the greater the number of possible empirical formulae or combinations of atoms that could produce that same nominal *m/z* signal.

5.4 Isotope Patterns

The relative intensity and position of isotope cluster peaks in a mass spectrum provide orthogonal information to the *m/z* data. Isotopes represent variants of a given element with the same number of protons and a different number of neutrons. The number and relative abundance of isotopes are unique to each element and together comprise the isotope pattern for a particular molecule in a mass spectrum. The relative abundance of each isotope also is a function of the number of each element

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in a molecule. Natural isotopic abundances depend on the source or provenance of a material and are influenced by variations of the isotopic abundances of its constituent elements. Because a unique elemental formula is consistently indicated by each peak coinciding with the *m/z* and relative abundance of each isotope in a molecular formula, the resulting isotopic fingerprint can be a powerful tool for the prediction of elemental composition.

Chlorine and bromine are examples of elements that produce prominent and easily recognizable isotope patterns in a mass spectrum. Chlorine atoms are characterized by the isotopes ^{35}Cl:^{37}Cl (approximately 3:1) and bromine atoms by unmistakable doublets (^{79}Br:^{81}Br = approximately 1:1) that enable easy identification of ions containing these atoms in a mass spectrum. Combinations of multiple Cl or Br atoms in a molecule yield characteristic isotope peaks that differ by two units and have relative abundances that can be calculated. For some types of HRMS (TOF in particular), the relative intensities of the isotopic cluster can be carefully defined. Because the relative intensity of the isotope cluster is exactly a function of the elemental composition of the molecule (that is easily calculated), isotope fitting of experimental data to theoretical intensity can help provide the correct formula.

Figure 5 presents the isotopic pattern in the molecular ion region for *n*-butyl benzenesulfonamide collected using ESI with different mass spectrometers, each having different mass resolution. With nominal mass (i.e., quadrupole mass spectrometer) resolution, one can clearly see the A + 1 (*m/z* 215) and A + 2 (*m/z* 216) peaks of the $[M + H]^+$ ion at *m/z* 214. The major contributor to the A + 1 peak results from the occurrence of versions of the molecule containing ^{13}C, and the major contributor to the A + 2 peak, is naturally occurring ^{34}S. With a higher-resolution mass spectrum, the more accurately calculated relative abundances of the A + 1 and A + 2 peaks could be used to estimate the number of carbon and sulfur atoms in the molecule, respectively. An examination of the FT–ICR mass spectrum illustrates the additional isotopic information that can be obtained under even higher-resolution conditions. This spectrum clearly shows that the A + 1 peak, seen as one peak with the other two mass spectrometers, also has contributions from other isotopes. At resolutions afforded by ICR or orbital-trapping mass spectrometers, the various contributors to the A + 1 peak can be observed, and one would get a more accurate estimate of the atoms present.

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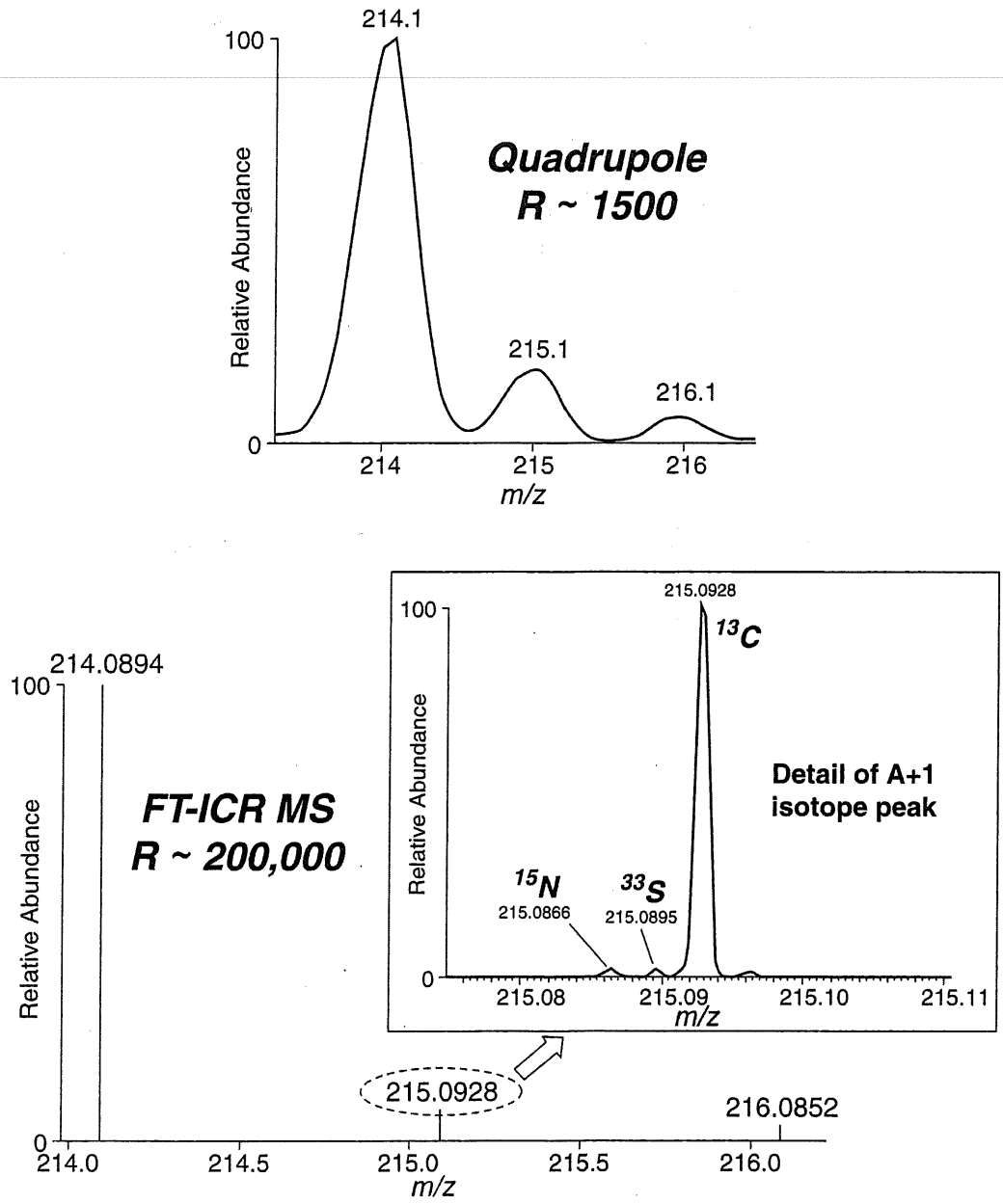

Figure 5. Partial ESI mass spectrum of *n*-butylbenzenesulfonamide under different mass resolution conditions.

Examination of isotope peaks also can reveal information regarding the charge state of an ion. For example, the difference between the A and the A + 1 isotope peaks is 1 *m/z* for a singly charged ion, a mass difference of 0.5 *m/z* denotes a doubly charged ion, and a mass difference of 0.3 *m/z* denotes a triply-charged ion. Thus, the correct charge state can be determined when only one peak or one set of isotope peaks is present, which supports the determination of MW and elemental composition. Because accurate mass and isotope fitting are orthogonal data that each separately can greatly reduce the number of possible empirical formulae for a given nominal mass, the information used together can reduce experimental data to a single or limited set of possible choices.

5.5 The Monoisotopic Ion

The monoisotopic ion is an important feature of a mass spectrum, and recognizing this ion is valuable in determining the elemental composition of a molecule or ion, particularly with accurate mass measurements. The monoisotopic mass of an ion or molecule is the exact mass calculated using the mass of the most abundant naturally occurring isotope of each constituent element. Therefore, the monoisotopic ion is represented by the isotopic peak composed of the most abundant isotopes of its elements. Importantly, MS observes the monoisotopic mass of the analyte, not its molecular weight (i.e., the average including

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contributions from heavy isotopes). Recognizing the monoisotopic ion peak can be straightforward, and some singly charged ions and elements present simple or easily recognizable isotope patterns. However, the combination of multiply charged ions and multiple elements that collectively contribute to complex isotope patterns occasionally can make determination of the monoisotopic ion peak quite challenging, particularly with polymers and biomolecules. In such cases the benefit of instrumentation that offers higher resolving power is readily apparent.

5.6 Fragmentation

Fragmentation of an organic molecule in a mass spectrometer and examination of the ions produced in the fragmentation process can provide detailed information about unique structural features such as functional groups, substituents, and connectivity. Fragmentation generally is the dissociation of an activated analyte ion. Observed fragmentations are quite diverse and can range from simple events such as loss of water or ammonia to highly complex pathways that involve adduction, multiple rearrangements, and atom migration. In MS, fragmentation is highly dependent on the type of instrumentation and operating conditions. Primary considerations include ionization source, collision zone conditions, and type of mass analyzer. To some extent, controlling these factors permits the selection and optimization of conditions that are conducive to generating desired or structurally diagnostic fragmentation.

EI fragmentation results entirely from unimolecular dissociation that occurs in high vacuum. Formation of a radical molecular ion ($M^{+\cdot}$) in an electron beam (typically 70 eV) is accompanied by redistribution and accumulation of energy into different vibrational modes, and as a result the molecular ion may undergo fragmentation. Fragmentation typically occurs by bond cleavage and produces an even-electron cation and a neutral odd-electron radical. The converse also can occur, albeit less frequently: an odd-electron radical cation is observed with an even-electron neutral fragment loss. Fragment ions can further dissociate to generate additional fragments. Generally, fragmentation of odd-electron ions may produce either odd-electron or even-electron ions, but even-electron ions fragment to other even-electron ions. The probability of cleavage of a particular bond in a molecule can be correlated with bond strength and the relative stability of the resulting fragment ions. Some general principles apply with regard to the presence of fragment ion peaks observed in EI mass spectra and can assist with interpretation. These principles have been thoroughly discussed in numerous texts on the topic and are not discussed here.

API and soft-ionization procedures predominantly produce ions such as $[M + H]^+$ or $[M - H]^-$ that exhibit limited fragmentation. API and other softer ionization procedures achieve fragmentation by activating ions in a variety of ways, depending on the type of instrumentation.

5.7 Biomacromolecules

Structural elucidation of biomacromolecules using MS offers a number of challenges that are not common among traditional drug substances described previously in the chapter. To start with, biomacromolecular therapeutics encompass many compounds with very different chemical properties that range from proteins and peptides to a variety of glycoconjugates, lipids, and even DNA and RNA complexes. To further complicate the matter, the molecular mass of biomacromolecules can range from under 1000 in the case of some bioactive peptides and lipids to well over 100,000 for vaccines, antibodies, and heparin complexes. Furthermore, many of these biologically derived substances exhibit considerable heterogeneity and thus may not be characterized as a single drug substance but instead as a mixture that is monitored for consistency from batch to batch using a variety of analytical methods.

This section provides guidance about MS characterization of biomacromolecules. Because of the complexity of this broad class of compounds, this section focuses on a few types of compounds to illustrate the benefits of MS, then comments briefly about how MS can be implemented for other biomacromolecular compounds.

5.7.1 PEPTIDES AND PROTEINS

Details of the process for characterizing proteins and peptides are provided in *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩ and are only summarized in this section to provide context.

5.7.1.1 Spectral interpretation: Mass spectral evaluation of proteins and peptides typically encompasses MS data collected on the intact compound(s) to establish the overall mass of the protein followed by tandem MS to generate information about the primary amino acid sequence of the protein or to characterize sites of protein modification. Interpretation of fragmentation spectra to generate the sequence information is a critical component of this approach, and thus some guidelines for the spectral interpretation of peptide fragments are provided in *5.7.1.2 Database searching*. Primary and secondary MS-based approaches have emerged as the standards for sequencing proteins by fragmentation and are known as bottom-up and top-down sequencing.

5.7.1.1 Bottom-up—This approach encompasses fractionation of individual peptides generated by proteolytic digestion and sequencing of the peptides by tandem MS. The complete protein sequence then is recapitulated by piecing together the individual sequences. To get complete coverage of the protein, it is necessary to do separate digestions with multiple proteases with different cleavage specificities to get overlapping sequence coverage to rebuild the overall sequence. This approach is currently the most common MS-based approach used to characterize protein therapeutics.

5.7.1.2 Top-down—Advances in MS-based technologies and particularly ECD and ETD fragmentation offer another approach for sequencing proteins, whereby the intact protein is fragmented to generate sequence information without prior need to digest the protein into smaller peptides. Current challenges with this approach include the need for purified proteins; difficulty in getting complete sequence coverage, particularly for larger proteins; the need for considerably larger quantities of proteins than for the bottom-up approach; and a requirement for a high-resolution instrument with ECD or ETD capabilities. However, during the development of proteins therapeutics the availability of large quantities of purified protein is a likely scenario, and with the continued advancement of MS technologies, top-down proteomics may offer a direct option for characterization of some proteins therapeutics in the future.

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5.7.1.2 Database searching: Because of progress in whole-genome sequencing and the computing power of predictive algorithms for the annotation of all possible genes, mRNAs, and proteins from genomic information, the characterization of unknown protein and peptide therapeutics rarely requires complete de novo sequence evaluation. Instead, the compound can be initially identified by digestion and sequencing by peptide fragmentation. This is possible because various fragmentation strategies (e.g., CID and ETD as described previously) produce gas-phase fragment ions from protonated peptides that represent consistent cleavage positions along the peptide backbone. The Roepstorff nomenclature for general peptide fragmentation along the peptide backbone is shown in *Figure 6A*. Peptide fragments that maintain a charge on the C-terminus are the *x*, *y*, or *z* ions, and those that maintain a charge on the N-terminus are known as *a*, *b*, or *c* ions. By optimizing the fragmentation energies and the collision gas to target only a single fragmentation event per protonated peptide molecule, analysts can use the collective fragmentation spectra from a given peptide to produce a set of fragment ions from the same series (i.e., *b* ions or *y* ions) that vary by the residue mass of each amino acid. In this manner they can derive the sequence. A schematic of a peptide fragmentation profile with a complete set of *y* ions is shown in *Figure 6B*. The complete set of *y* ions allows the sequence to be determined from the mass difference between the ions that correspond to residue masses for each amino acid. With the development of complete databases of many genes, transcripts, and proteins, a variety of commercially available computer algorithms have been developed to match the fragmentation masses with protein sequences in databases, along with false discovery rates and confidence probabilities. As a result, confirmation of protein sequences for known proteins can be fully automated for data collection and evaluation.

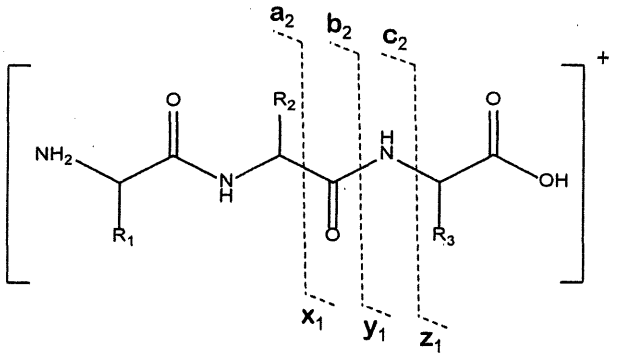

Figure 6A. Peptide fragmentation nomenclature.

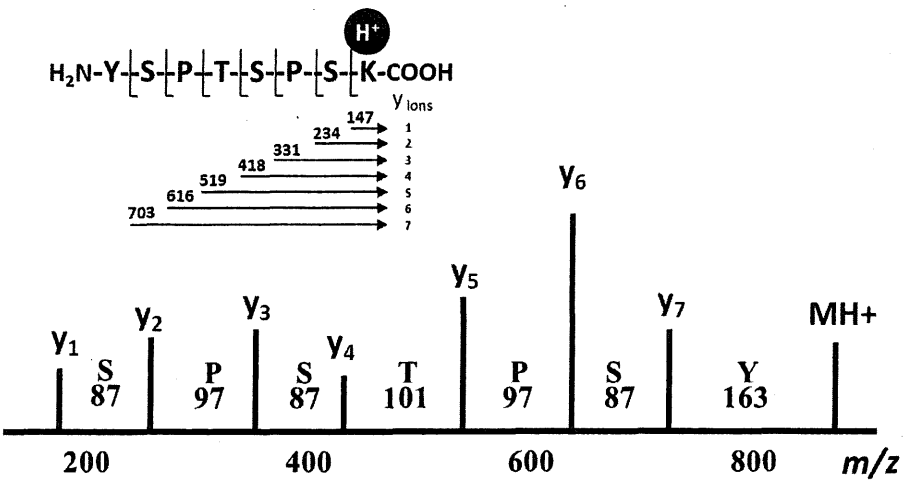

Figure 6B. Peptide fragmentation sequence.

5.7.1.3 De novo sequencing approaches: When a protein is completely unknown, MS-based fragmentation can be used to generate sequence information as described. The primary challenge is the inability to always get complete, unambiguous sequence information for some peptides by MS fragmentation because of the inability to assign all amino acids from a given fragmentation spectrum without a template of known sequences. In cases such as these, a combination of chemical sequencing using Edman degradation and MS may be necessary. Alternatively it may be necessary to digest and sequence the protein with multiple proteases in order to generate overlaying sequences to ensure the reliability of the overall sequence assignments.

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5.7.2 GLYCOCONJUGATES

The analysis of drug substances in this class is discussed in *Glycoprotein and Glycan Analysis—General Considerations* ⟨1084⟩. Thus this section addresses only the application of MS to the characterization of protein glycosylation.

Many biopharmaceuticals are glycoproteins for which glycosylation plays critical roles in maintaining function, stability, and solubility. The most common glycosylations are *N*-linked and *O*-linked glycosylation in addition to other glycosylation modifications such as GPI anchors. *N*-glycosylation refers to glycan modification on the asparagine side chain, and *O*-glycosylation occurs to the side chains of serine and threonine. MS analysis of protein glycosylation can be performed on three different levels: intact proteins, peptides, and glycans. This section provides a brief overview of glycoprotein analysis.

5.7.2.1 Intact protein analysis: For proteins with small size or relatively simple glycosylation, direct MS analysis of intact protein provides a mass profile that reflects the protein's primary sequence and major modifications, including glycosylation. This type of experiment usually is performed using TOF analyzers or analyzers with higher mass resolution equipped with ESI or MALDI ionization. MALDI forms mainly singly charged ions and requires MS analyzers with a wide m/z range for measurement of intact protein mass (e.g.,TOF). ESI–MS is commonly used, because it leads to the formation of multiply charged protein ions in the m/z range of 700–5000, resulting in better mass accuracy in protein mass measurement (<100 ppm when coupled with a high-resolution mass sepctrometer). MS analysis in many cases can resolve protein peaks by their glycoform distribution because of the mass differences (e.g., hexose 162 Da, HexNAc 203 Da, and *N*-acetyl neuraminic acid 291 Da). The mass information can lead to identification of the monosaccharide composition of the glycoforms. The intensity of the intact protein peaks can be used to roughly estimate the glycoform distribution on the protein. For monoclonal antibodies modified mainly by neutral *N*-glycans, quantification of glycosylation distribution by intact protein analysis appears to be comparable with measurement using glycan separation methods. Antibodies containing multiple glycosylation sites can be enzymatically cleaved into major domains before MS analysis to gain more specific information about the position of the glycosylation on the intact protein.

5.7.2.2 Peptide analysis: For proteins with complicated glycoforms and multiple glycosylation sites, site-specific glycosylation information usually is desirable and is achieved by using a bottom-up proteomics approach via analysis of peptides generated by enzymatic digestion. The peptides are separated by reversed-phase LC followed by ESI–MS/MS analysis. MS–MS fragmentation can be performed using CID or ETD. In CID–MS/MS, the peptides can be identified to be glycosylated peptides by the detection of oxonium ions in the peptide MS–MS spectra [e.g., m/z 163 (hexose), 204 (HexNAc), 292 (sialic acid), and 366 (hexose-HexNAc)]. However, CID–MS/MS spectra of glycosylated peptides usually are dominated with fragmentation from the glycan with minimal fragmentation between the amino acid residues of the peptide backbone, thus hampering the ability to identify the protein sequence. ETD–MS–MS spectra of glycosylated peptides contain mainly fragmentation between the amino acids (e.g., *c*- and *z*-type peptide backbone cleavages), while the attached glycans remain intact. ETD–MS–MS provides information about peptide sequence, glycosylation site, and the glycan mass of the glycosylated peptides. CID– and ETD–MS–MS may be used alternatively in the same LC–MS analysis of complicated protein digests, which may provide the information necessary for identification of sequence, glycosylation sites, glycan mass, and glycan branching. *O*-glycosylated peptides also can be analyzed by using similar approaches. Peptide glycosylation can be identified by observation of oxonium ion such as m/z 204 in CID–MS–MS, and the peptide sequence is identified by ETD–MS–MS when the *O*-glycan moieties remain attached to the peptide.

5.7.2.3 Glycan analysis: *N*- and *O*-glycans can be enzymatically or chemically released from proteins followed by MALDI–MS or ESI–MS analysis either directly or after derivatization with chemical tags for enhancement of their ionization.

The direct MALDI–MS analysis of released glycans can be performed in both positive and negative mode for the detection of neutral and acidic glycans, respectively. The commonly used matrices include 2,5-dihydroxybenzoic acid (DHB) and 2′,4′,6′-trihydroxyacetophenone. The neutral glycans usually are detected as $[M + Na]^+$ in positive ion MALDI–MS. ESI–MS analysis of glycans suffers from poor ionization of the glycans and thus are used only following chemical derivatization. For example, derivatization of released glycans from glycoproteins can be performed on the reducing end of glycans by reductive amination using fluorescent tags such as 2-aminobenzamide. The labeled glycans are analyzed using hydrophilic interaction chromatography followed by ESI–MS analysis in positive ionization mode. The molecular mass of the glycans can be determined, and subsequent MS–MS analysis can provide structure information about the glycans. Permethylation is another important glycan derivatization method that neutralizes acidic glycans and allows both neutral and acidic glycans to be detected by MS in positive ion mode. MS/MS spectra of glycans contain ions from two major types of cleavages, as illustrated in *Figure 7*. Glycosidic cleavage occurs at the bonds between two sugar rings and results in *B*, *C*, *Y*, and *Z* ions, which provides important information regarding monosaccharide sequence and glycan branching. Cross-ring cleavages (shown in *Figure 7* by dashed lines) involve rupture of two bonds on the same sugar ring and lead to *A* and *X* ions that assist in identification of the type of sugar linkages in the glycans. With increasing understanding of glycan fragmentation, the identification of glycan from its MS–MS spectrum now is possible via database searches using recently developed computational algorithms.

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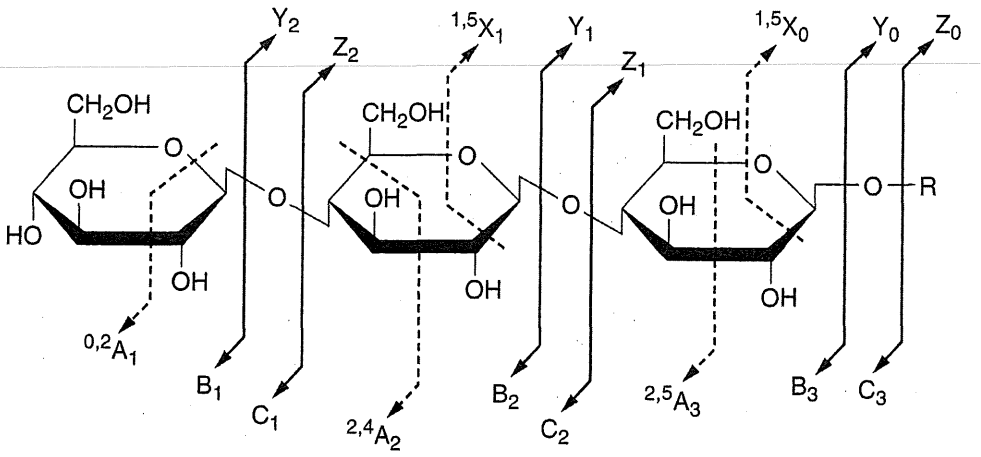

Figure 7. Nomenclature for carbohydrate fragmentation. Source: Adapted from Domon B and Costello CE (1988). *Glycoconj. J. 5*, 397–409.

5.7.3 VACCINES

Vaccines present a unique challenge because often they include complex mixtures of proteins, polysaccharides, and lipids in the form of intact bacteria or viruses, outer membrane vesicle preparations, or combinations of recombinant proteins. General information regarding bacterial vaccines is available in *Vaccines for Human Use—Bacterial Vaccines* ⟨1238⟩. Quality control of such mixtures typically relies on characterization by SDS–PAGE, SEC, amino acid or monosaccharide compositional analyses, RP–HPLC, and other analytical methods. However, to date MS has not been used as a primary QC method even though it is frequently used for confirmation of identity and characterization of impurities. Because of the many advantages of MS described in this chapter and its widespread use in the characterization of biotherapeutics and vaccines that contain complex protein mixtures, MS and MS–MS offer the ability to identify product and impurity simultaneously, and likely will be adapted in routine QC environments in the future. Additionally, when a complication occurs (e.g., discrepancy between assays, activity changing after formulation, or posttranslational modification), the quantitation of the recombinant protein (within a complex proteome) or key antigen in a complex protein vaccine via a highly specific procedure such as MS may be desirable. The use of isotope dilution MS–MS in combination with multiple reaction monitoring scans can provide highly specific and accurate quantitation of protein in complex samples.

5.8 Monograph Methods for Qualitative Identification

The discussions in this chapter and in *Mass Spectrometry* ⟨736⟩ covering the qualitative capabilities of MS provide strong technical justification for its use as part of a compendial identification strategy for a wide range of molecular classes. The structural information content (e.g., molecular mass, empirical formula, structurally significant fragmentation, and characterization of biomolecular modifications such as glycosylation) has been and will be an integral part of identification strategies. One or more specific aspects of mass spectral information can be combined to increase the specificity and reliability of the proposed test. Development of the MS portion of the compendial identification test should strive to provide unique and complimentary information for use with other identification tests intended in the monograph. Method development also should consider the availability of a reference standard for successful mass spectrometric identification.

6. QUANTITATIVE ANALYSIS

MS is useful for the quantitative determination of actives or impurities in a drug substance or a drug product because of its selectivity and sensitivity. The former typically comes into play when target compounds reside in complex matrices (e.g., natural or formulated), and the latter is a benefit when low-level impurities must be measured. Selectivity is achieved in MS-based methods by the combination of well-characterized attributes of the target compound, including molecular mass, intrinsic ionization characteristics, and fragmentation with additional data such as chromatographic retention time. In addition to chromatographic selectivity, use of a chromatographic inlet helps isolate the target compound from other chemical entities within the sample that could create problematic artifacts (e.g., ionization suppression) if they were introduced concomitantly.

This general approach to sample analysis (i.e., chromatography coupled with MS) typically introduces the target compound into the mass spectrometer as a well-defined peak (ideally, Gaussian) that can be reproducibly integrated for optimal quantification. While other forms of sample introduction (e.g., flow injection and direct-insertion procedures such as MALDI) potentially can be employed and may be necessary in some applications, chromatographic introduction systems are more common and are preferred for MS quantification.

Although various mass spectrometer types can be used, the quadrupole and triple quadrupole, in particular, are most commonly employed for quantitative analysis. These instruments provide a combination of specificity, sensitivity, stability, and linear response, which is essential for accurate measurement of target compounds, particularly within complex formulations. For these reasons, the balance of this quantification discussion emphasizes the use of chromatographic separations in concert with quadrupole MS detection.

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6.1 MS Quantitation Overview

Employing a chromatographic inlet, analysts introduce a sample solution via injection onto an appropriate chromatographic column, and a flowing carrier stream (gas or liquid mobile phase) advances the target compound(s) and other matrix components through the column at various rates that are determined by strength of the carrier (gas characteristics for GC; organic, along with mobile-phase modifiers, for HPLC), temperature, and affinity for a stationary phase (see ⟨621⟩). Pressurized or near-atmospheric-pressure chromatographic effluent is passed through an appropriate interface into the vacuum environment of the mass spectrometer. In this manner, chemical components within the sample enter the mass spectrometer as chromatographic peaks that are subject to ionization as defined by the chosen ionization mode and according to the propensity of each chemical to become ionized under those experimental conditions.

In the case of single-quadrupole MS detection, the mass analyzer is set to pass to the detector an ion with an *m/z* value that is characteristic of the target compound. In this selected ion monitoring (SIM) mode, a chromatographic profile for a single *m/z* value is created. This overall process yields a chromatographic peak at the retention time expected for the target compound. When integrated, the area under this peak leads to a measure of target compound concentration in the original sample. Accuracy in an MS-based quantitative assay is achieved by the proper use of a well-characterized reference standard and, ideally, an appropriate internal standard, as described in *6.2.1 Internal Standards*.

For many analyses, the combination of chromatographic separation and SIM MS analysis will provide sufficient selectivity to yield an analysis, which is sufficiently free of chemical interference to meet the measurement need. However, in some situations, it may be necessary to utilize the additional selectivity provided by tandem MS detection that commonly is provided by a triple-quadrupole instrument. The need can be particularly acute in Category II analytical procedures for which a trace impurity must be measured within a complex formulation.

The relative benefit of MS/MS detection is illustrated in the extreme example presented in *Figure 8*. The antitussive dextromethorphan (Dex) is present in a plasma extract at 100 pg/mL. This extract is first analyzed by LC–MS using ESI–SIM at *m/z* 272 (protonated molecule). In this experiment the SIM chromatographic peak corresponding to Dex is barely discernible above the relatively high background noise. Alternatively, a triple quadrupole can be employed to set up a selected reaction monitoring (SRM) detection scheme for added selectivity. SRM is a special case of product ion MS–MS in which the second mass analyzer is tuned to pass only a selected fragment ion corresponding to Dex (*m/z* 147 in this example). A marked enhancement in selectivity, and therefore signal-to-noise ratio, is evident and typically leads to improved performance for quantitative analytical methods.

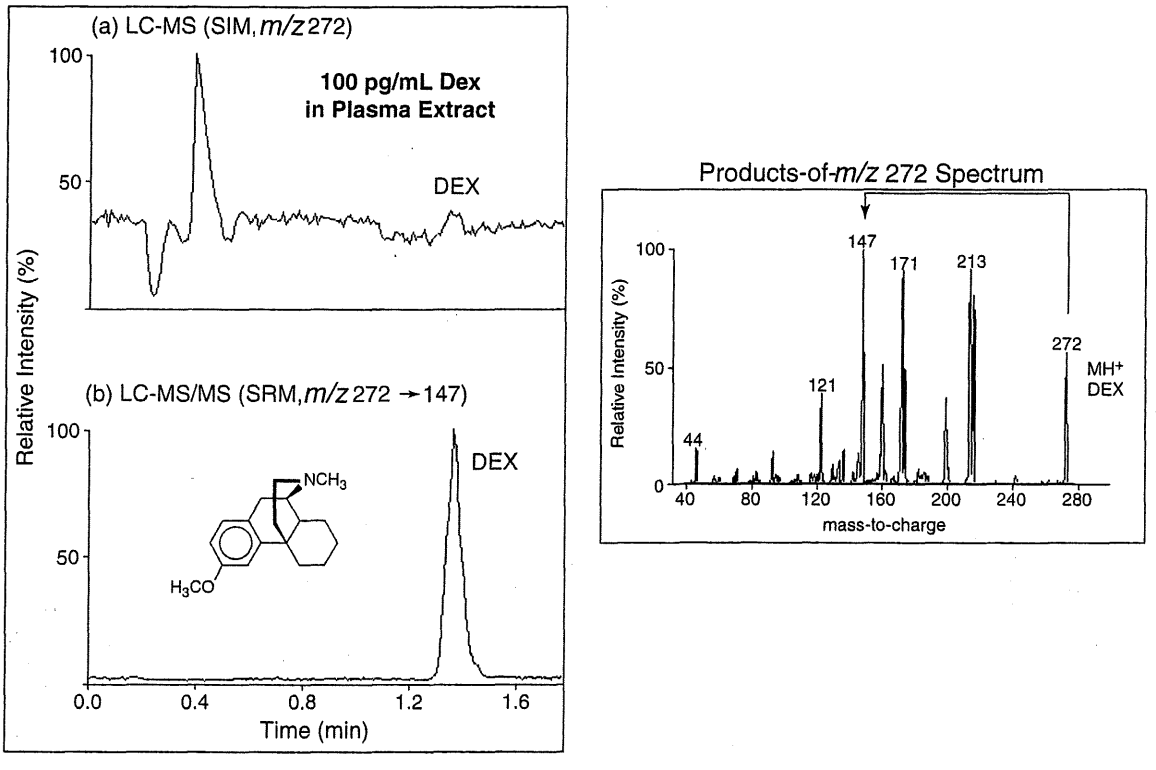

Figure 8. MS (SIM) versus MS–MS (SRM) for quantitative methods.

6.2 Quantification Considerations and Procedures

Methods suitable for Category I and Category II applications require the use of a reference standard for each target compound. Quantification of a target compound in an appropriately prepared test sample is achieved by relating its MS

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response (e.g., SRM-derived chromatographic peak area) to that of similarly prepared reference standards spanning the required concentration range (see *Mass Spectrometry* ⟨736⟩, *Validation and Verification of Mass Spectrometry Analytical Procedures*).

6.2.1 INTERNAL STANDARDS

For optimal accuracy and precision, internal standards should be employed at a consistent concentration in both the test samples and reference standards. Internal standards compensate for small sample-to-sample differences in dilution volume or loss of material during sample processing. They also compensate for analysis-to-analysis fluctuations in MS response or sensitivity drift over the course of batch analyses, both of which occur in all MS-based methods. In practice, selective detection schemes are set up to monitor both the target compound (analyte) and its corresponding internal standard. The key instrumental measure for each standard and test sample is, therefore, a ratio of the analyte-to-internal standard chromatographic peak areas. Internal standards can be either structural or stable-isotope-labeled analogs of the target compound. The former may be adequate and often has the advantage of lower cost and availability. The isotopically labeled (e.g., ^{2}H, ^{13}C, or ^{15}N) alternative offers superior method performance, because it is essentially identical to the analyte in chemical and physical properties, with the exception of greater molecular mass. The only requirements are that the isotopic labels are not subject to chemical exchange during storage or sample preparation, are retained in the MS ionization process, and provide a sufficient molecular mass shift (typically 3+ Da using multiple labels) versus the analyte to ensure negligible cross-talk (interference) between analyte and internal standard signals (because of natural isotopes and variable m/z resolution across different mass analyzers). In addition, care must be taken to ensure that the internal standard contains inconsequential levels of unlabeled target compound impurities that potentially could bias results. Suitability of the internal standard is confirmed after successful completion of the analytical procedure validation.

6.2.2 DATA ACQUISITION

For accurate integration of analyte and internal standard chromatographic peaks, the data acquisition parameters (e.g., sampling rate, scan range, or masses monitored) must provide a sufficient number of intensity samples across the peak width. The number of samples may vary depending on method performance requirements (see *Table 1* in general chapter ⟨736⟩) and practical considerations related to chromatography conditions, mass spectrometer type, and the number of analytes and internal standards monitored. For example, for single-analyte assays on quadrupole-based instruments, analyte and internal standard SIM or SRM detection schemes are alternately sampled throughout the analytical run. With modern quadrupole instruments, dwell times of 100 ms or less per sampling point are readily achieved and should result in at least eight samples across each peak (this is the suggested minimum for good quantitative representation of the peak).

6.2.3 CALIBRATION

A calibration curve is created by plotting analyte-to-internal standard peak area ratios versus concentration for the reference standards analyzed. For the somewhat narrow calibration range required for Category I and Category II measurements, a simple (nonweighted) linear fit is sufficient to define this response versus concentration function. However, $1/x$ or $1/x^2$ weighting also can be used, as well as nonlinear (e.g., quadratic) functions, as supported by appropriate and provided validation data.

The calibration concentration range is selected based on instrument sensitivity and the required method performance attributes. In general, the lowest concentration should yield sufficient signal for reproducibility, and the highest concentration ideally should be within the linear response range of the MS system. In extreme cases, the upper end of the linear response range may result from limitations of the mass analyzer or detector (e.g., as with TOF and ion trap MS instruments, but much less so with quadrupole mass analyzers). More often, loss of linearity results from diminishing ionization efficiency at higher concentrations. This is a common consideration with, for example, electrospray ionization where high analyte concentrations may increasingly deplete available ionizing adduct ions, leading to a less-than-proportional increase in detected signal with increasing concentration. This so-called self-suppression phenomenon can be somewhat compensated by the use of a stable-isotope-labeled internal standard that co-elutes with the analyte and, therefore, experiences similar proportional suppression. However, best-method performance is achieved when calibration falls within the linear range.

6.2.4 TEST SAMPLE PREPARATION

The scope of acceptable sample preparation procedures for quantitative MS analysis is broad and depends on many factors, including the analyte chemical class, the composition and complexity of the matrix, the target analyte concentration, and the type of analytical instrumentation available. If appropriate, the preferred approach is simple dissolution followed by internal standard addition and then dilution to a target concentration within the calibration range. This simplicity minimizes the potential for analyte recovery issues and contamination or other procedural errors that may compromise the robustness of more complex methods. Provided sufficient instrument sensitivity, optimal method performance typically is achieved with greater dilution factors (lower analyte concentrations in the prepared sample). This approach not only avoids self-suppression issues but also minimizes the potential for ionization suppression due to co-eluting matrix components.

More complex sample preparation procedures may be required to address issues related to the chemical class or concentration of the analyte, as well as the nature of the matrix. In such cases, the use of an internal standard (stable-isotope-labeled standards in particular) is almost essential to ensure adequate method performance. For best results, the addition of the internal standard should take place at the earliest practical stage of sample preparation (e.g., immediately following test sample dissolution).

For example, procedures such as liquid–liquid and solid-phase extraction may be required to enrich (concentrate) target compound(s) for greater assay sensitivity or for isolation from high levels of ionization-suppressing components within the matrix (e.g., salts or other excipients). Chemical derivatization of certain functional groups of a target compound may be desirable to stabilize the sample (e.g., for GC–MS analysis), improve method chromatographic performance, or enhance

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intrinsic sensitivity for detection (e.g., for ESI). Although such procedures add complexity, they are acceptable provided the overall analytical procedure can be properly validated.

A special case of complex sample preparation that may be required for quantification of macromolecular compounds such as vaccines and biotherapeutics involves the intentional degradation of the target molecule(s) into smaller entities that are more amenable to quantitative MS analysis. For example, partial degradation of a protein therapeutic may be achieved by hydrolytic (chemical) or enzyme-based (e.g., tryptic) digestion to yield peptide fragments that are characteristic of the protein. These smaller molecules can be analyzed by using principles described in this section (including the use of stable-isotope-labeled internal standards of each targeted peptide), and the results can be correlated to determine the concentration of the original target protein. Further, by measuring multiple peptides derived from the protein of interest, analysts can achieve qualitative (identity) verification of the protein by means of the same method. Of course, this indirect approach usually requires some degree of isolation and qualitative identification of the macromolecule to ensure that the subsequent quantitative measure of the representative peptides is relevant.

7. EMERGING MS APPLICATIONS

7.1 Drug Product Authentication and Contamination Detection

Counterfeit and contaminated or adulterated pharmaceutical materials are a threat to patient safety, consumer confidence, and product security and are therefore of concern to pharmaceutical manufacturers and regulatory agencies. Because increases in the incidence of counterfeited and contaminated pharmaceutical materials have elevated concerns about public health safety and product security, numerous measures have been employed to detect counterfeit and contaminated drug products and to ensure supply chain integrity. Because of its sensitivity and specificity, MS is an effective analytical tool for broad application in the determination of authenticity, source, or contamination of drug products. Approaches include verifying the identity of the active ingredient(s), determining and comparing impurity profiles, and isotopic characterization.

7.1.1 IDENTIFICATION AND/OR VERIFICATION OF THE ACTIVE INGREDIENT

Counterfeit drug products may contain multiple or different active ingredients or no active ingredient at all. GC–MS, LC–MS, or direct-infusion MS procedures can provide a verification of the presence or absence of an active ingredient or the identity of other active ingredients that may be present. Counterfeit drug products containing multiple active ingredients or very high levels of a single active ingredient (superpotent samples) typically are of greater concern to human safety than those that contain little or none. In cases such as these, MS can provide positive, unequivocal proof of identity, as well as quantitative information sufficient to pursue those responsible for producing the counterfeit products.

7.1.2 IMPURITY PROFILING

Impurity profiles can indicate contamination of a drug product or can distinguish an authentic product from a counterfeit. Impurity profiles serve as fingerprints for a drug substance or formulated product from a particular source. Hyphenated techniques such as LC–MS and GC–MS can be used to resolve and identify process-specific impurities that are characteristic of a particular synthetic route, which can be useful in determining the origin and distribution of counterfeit materials. Variations in the impurity profile of the authentic material and changes in the profile with time and process conditions must be considered when analysts use impurity profiles for identification or as indicators of potential contamination. With suspect counterfeit products, similarity of impurity profiles may not necessarily indicate a suspect sample is authentic. In such cases, additional investigation using orthogonal procedures may be necessary.

7.1.3 ISOTOPIC CHARACTERIZATION

Stable isotopes of elements such as carbon, nitrogen, hydrogen, and oxygen occur naturally in pharmaceutical products and raw materials. Isotopic ratios principally depend on starting materials and processes used to produce drug substances and drug products and can be highly specific for a given batch. Therefore, isotope ratios can define an isotopic fingerprint for a given material, and observed differences in specific isotope ratios between batches can be used to authenticate materials or indicate potential contamination. Isotope ratio mass spectrometry (IRMS) is a powerful tool for pharmaceutical authentication or detection of potential contamination in drug products and raw materials. IRMS affords a high level of specificity, accuracy, and precision in the determination of certain elemental (e.g., carbon, nitrogen, oxygen, and hydrogen) isotope ratios in drug substances or products. Because specific isotope ratios can be influenced by multiple factors such as location, climate, process differences, and source and quality of raw materials, IRMS is able to provide information that is not only useful in authenticating a drug product but also in indicating the origin of a drug product or material. This is particularly important in determining whether distributed counterfeit drug products obtained at discrete locations originate from the same manufacturing process or when linking a diverted product with its original manufacturing site. *Figure 9* illustrates subtle but notable differences between authentic drug product samples from three global manufacturing sites and overt differences between authentic and three counterfeit drug products. Additionally, IRMS approaches authentication from a postproduction perspective and does not involve marking or altering the drug product, as may be required by other pharmaceutical authentication technologies.

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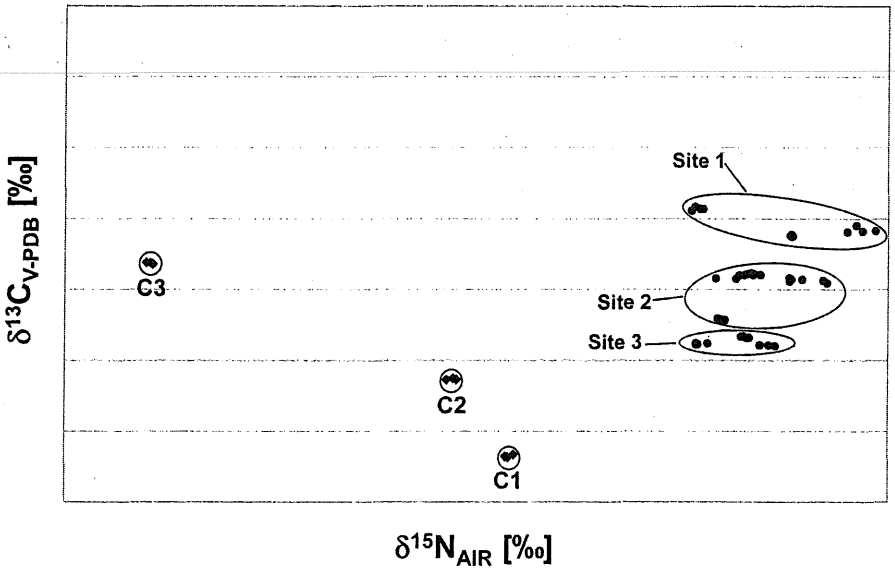

Figure 9. Bivariate plot of stable isotopic composition ($\delta^{13}C$ versus $\delta^{15}N$) of authentic drug product from three global manufacturing sites (Sites 1–3) and three counterfeit drug products (C1–C3).

⟨1761⟩ APPLICATIONS OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

PRINCIPLES OF NMR

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique based on the magnetic properties of certain atomic nuclei. NMR is similar to other types of spectroscopy in that absorption of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs from other types of spectroscopy because the discrete energy levels between which the transitions take place are created by placing the nuclei in a magnetic field of strength H_0. Although the initial field strength of the applied field is H_0, when the sample is inserted into the magnet, the field strength throughout the sample becomes B_0, defined as follows:

$$B_0 = \mu_s H_0 \text{ [1]}$$

in which μ_s is the magnetic susceptibility of the sample.

Atomic nuclei behave as if they were spinning on the nuclear axis. The angular momentum, ρ_0, of the nucleus is characterized by a spin quantum number (I). The maximum observable component of the angular vector, ρ, is

$$\rho = Ih/2\pi = I\,\overline{h} \text{ [2]}$$

in which h is Planck's constant and $\overline{h}$ is modified Planck's constant.

Table 1 shows the values of I as a function of the mass number and the atomic number.

Table 1. Nuclear Spin Values as a Function of Mass and Atomic Numbers

Mass Number	Atomic Number	Nuclear Spin (I)
Odd	Even or odd	1/2, 3/2, 5/2 ...
Even	Even	0
Even	Odd	1, 2, 3 ...

The angular momentum creates a magnetic moment, μ, which is parallel to and directly proportional to ρ.

$$\mu = \gamma\rho = \gamma I\overline{h} \text{ [3]}$$

where γ is the magnetogyric ratio and is a constant for all nuclei of a given isotope, regardless of their position in a molecule.

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Nuclei that have a spin quantum number $I \neq 0$, when placed in an external uniform static magnetic field, align with respect to the field in $(2I + 1)$ possible orientations. Thus, for nuclei with $I = 1/2$, which includes most isotopes of analytical significance (*Table 2*), there are two possible orientations, corresponding to two different energy states. The energies of these two states are $\pm \mu B_0$, and their separation is

$$E = \mu B_0 - (-\mu B_0) = 2\mu B_0 \quad [4]$$

with more nuclei populating the lower energy state ($-\mu B_0$) than the higher energy state ($+\mu B_0$). The populations are in accordance with the Boltzmann distribution:

$$N_+/N_- = \exp(-E/kT) \quad [5]$$

where N_+ and N_- are the populations of the high and low energy states, respectively; k is the Boltzmann constant; and T is the temperature in K.

A nuclear resonance is the transition between these states, and upward as well as downward transitions are possible. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the B_0 axis. The precessional angular velocity is often referred to as the Larmor frequency, ω_0, and is related to B_0:

$$\begin{aligned} E = h\nu &= 2\mu B_0 \\ &= 2\gamma I \bar{h} B_0 \\ &= \gamma \bar{h} B_0 \\ \nu &= \gamma B_0 / 2\pi \\ \omega_0 &= \gamma B_0 \end{aligned} \quad [6]$$

If energy from an oscillating radio-frequency (rf) field is introduced, then resonance is achieved when the rf frequency is the same as the precessional angular velocity. Although the probability of an upward transition is equal to that of a downward transition, more upward transitions take place than downward transitions because N_- is greater than N_+. Hence, an overall absorption of energy takes place. As shown in *Table 2*, the resonance frequency of a nucleus increases in direct proportion with the increase of the magnetic field strength.

Table 2. Properties of Some Nuclei Amenable to NMR Study

				Resonance Frequency (MHz) at		
Nucleus	*I*	Natural Abundance (%)	Sensitivity	1.4093 T[a]	7.0463 T	11.7440 T
^{1}H	1/2	99.98	1.00	60.000	300.000	500.000
^{13}C	1/2	1.108	0.0159	15.087	75.432	125.721
^{19}F	1/2	100	0.83	56.446	282.231	470.385
^{31}P	1/2	100	0.0663	24.289	121.442	202.404
^{11}B	(3/2)	80.42	0.17	19.250	96.251	160.419

[a] T = tesla: 1.4093 T = 14.093 kilogauss.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the upper and lower energy states (0.08 Joules at 1.5 to 2.0 T field strength), which results in a population difference between the two levels of only a few parts per million. Another important aspect of the NMR phenomenon, with negative effects on sensitivity, is the long lifetime of most nuclei in the excited state. Long lifetimes affect the design of the NMR analytical test, especially in repetitive pulsed experiments. Simultaneous acquisition of the entire range of resonant frequencies instead of frequency-swept spectra can give increased sensitivity per unit time.

All characteristics of the signal–chemical shift, multiplicity, linewidth, coupling constants, relative intensity, and relaxation time contribute analytical information. The analytical usefulness of NMR arises from the observation that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field associated with a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. The latter field is generally opposed to the external field and lowers the overall field strength at the nuclear site. The phenomenon is termed *shielding*. Hence, the more shielded nuclei have lower Larmor frequencies.

It is not convenient to accurately measure the absolute values of transition frequencies, as is done with other spectroscopic procedures. However, it is convenient to accurately measure the difference in frequencies between two resonance signals. The position of a signal in an NMR spectrum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called *chemical shift*, which may be expressed in units of magnetic field or in frequency units that are readily interconverted by the equation for the resonance condition, *Equation 6*. This equation shows that when the separation is expressed in frequency units, it is directly proportional to the field strength. It is more convenient, therefore, to express the chemical shift in terms of the dimensional unit δ, which is independent of the field strength, and is defined by

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$$\delta = (\nu_{SS} - \nu_{RS})/\nu_0 \quad [7]$$

where ν_{SS} is the test substance resonance frequency, in Hz; ν_{RS} is the reference resonance frequency in Hz; and ν_0 is the instrument frequency, in MHz. When ν_0 is expressed in units of MHz, then *Equation 7* is expressed in units as parts per million (ppm). Hence, it is common to use the unit ppm to express the chemical shift difference between the resonance peak of the test substance and that of the reference.

By using this equation, one can use (with appropriate caution) the chemical shift of any known species (such as the residual ^{1}H-containing species in a deuterated solvent) as a chemical shift reference. This equation, now in common use, is applicable to nearly all nuclei.

Tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one peak (which is more shielded than most signals), and is volatile, which allows ready specimen recovery. Either 2,2,3,3-d_4 sodium 3-(trimethylsilyl)propionate (TMSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) is used as an NMR reference for aqueous solutions. The resonance frequency of the TMSP or DSS methyl groups closely approximates that of the TMS signal. Where the use of an internal NMR reference material is not desirable, an external reference may be used, such as a reference standard in a separate NMR tube.

Conventional NMR spectra are shown with shielding increasing and chemical shift decreasing from left to right because less shielded nuclei have higher Larmor frequencies than do more shielded nuclei. Resonances on the left are said to be deshielded (i.e., they show lower electron density). Resonance peaks appearing at the right are termed more shielded (i.e., they show greater electron density) than those appearing at the left. Resonances from the more shielded and the less shielded nuclei often are inappropriately called the high-field or upfield peaks and the low-field or downfield peaks, respectively, as a result of the outdated method of acquiring data by sweeping the magnetic field. Today, the overwhelming majority of spectra are acquired on a pulsed Fourier transform (FT) spectrometer, which sweeps neither the magnetic field nor the transmitter frequency. Therefore, it is more appropriate to refer to resonances at the left side of the spectrum as high-frequency or deshielded resonances and those on the right as low-frequency or shielded resonances.

The coupling between two nuclei can be described in terms of the spin-spin coupling constant, *J*, which is the separation (in Hertz) between the individual peaks of the multiplet. When two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting mutiplets are equal. Furthermore, *J* is independent of magnetic field strength.

Coupled spin systems are usually referred to as weak or strong. These terms depend on the separation of the Larmor frequencies of the coupled nuclei compared to the coupling constant between them. Both of these values are easily measured from the spectrum. For a weakly coupled system, the separation expressed in Hz ($\Delta\nu$) is large compared to *J*, which is always expressed in Hz. Thus, the ratio of the two is dimensionless. For a weakly coupled system, the ratio is large. Typically, spectroscopists consider a ratio above 10 to be weak. Only weakly coupled spin systems produce first-order spectra, which are comparatively easy to analyze. The number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by $2nI + 1$, where *n* is the number of identical nuclei on adjacent groups that are active in splitting and *I* is the spin of those nuclei causing the splitting. For protons this becomes $(n + 1)$ peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion $(a + b)^n$. These coefficients can conveniently be found by use of Pascal's triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. Two examples of first-order spectra arising from weak coupling are shown in *Figure 1*.

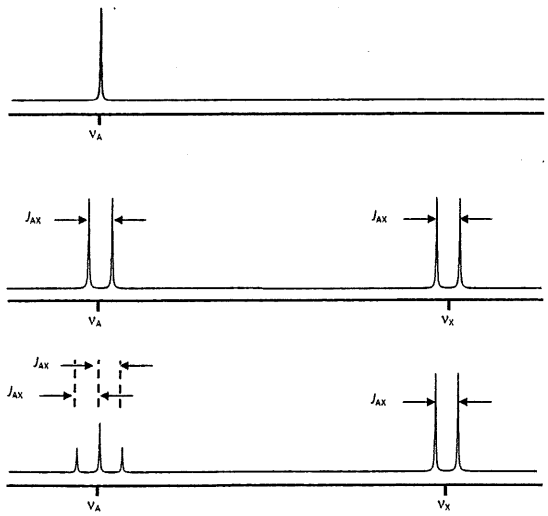

Figure 1. Diagrammatic representation of simple spectra resulting from weakly coupled spin systems.

Coupling may occur between ^{1}H and other nuclei, such as ^{19}F, ^{13}C, and ^{31}P. This type of coupling can frequently be observed between nuclei separated by 1–5 bonds.

Magnetically active nuclei with $I \geq 1$, such as ^{14}N, possess a nuclear quadrupole moment, which produces line broadening of the signals from neighboring nuclei.

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Another characteristic of an NMR signal is its relative intensity, which has wide analytical applications. In carefully designed experiments, the area or intensity of a signal is directly proportional to the number of protons that give rise to the signal. As a result, NMR can be used for quantitation (see *Quantitative Analysis* in this chapter and in *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis*. NMR spectra may contain spinning side bands, peaks that appear symmetrically located around each signal. These signals are due to the failure to optimize the off-axis (*x* and *y*) shims. The homogeneity of modern superconducting magnets, coupled with computer shimming techniques, reduce the need for sample spinning and completely eliminate these sidebands.

NMR SPECTROMETERS

Introduction

NMR spectrometers have evolved since the first commercial instrument, a Varian HR-30 that operated at 30 MHz, was produced in 1952. Initially NMR spectrometers used a data acquisition technique known as continuous wave (CW), which was based on sweeping the magnetic field. The limitations of a CW spectrometer include low sensitivity and long analysis time.

Today's spectrometers operate at frequencies up to 1 GHz and apply an rf pulse to the sample to produce a time-domain signal known as a free induction decay (FID), which is then Fourier transformed into a frequency-domain signal. This technique is known as FT NMR spectroscopy. Current NMR spectrometers are composed of several key components: the magnet, the probe, the console, and the computer.

The instruments are described by the approximate resonance frequency of the ^1H resonance, e.g., 600 MHz, or by their field strength, e.g., 14.1 T.

The Magnet

Until the early 1970s, NMR magnets were either ferromagnetic-core electromagnets or permanent magnets that operated at field strengths of 1.41–2.35 T, corresponding to ^1H resonance frequencies of 60, 80, 90, and 100 MHz. The first NMR magnets based on superconducting magnets were introduced in the 1960s and allowed access to much higher field strengths that currently are as high as 23.5 T (1 GHz).

The superconducting magnet is the most expensive component of an NMR spectrometer and can cost up to several million dollars. These magnets consist of miles of Nb_3Sn or NbTi wire. When these materials are wound into a solenoid that is immersed in liquid helium at 4.2 K, they become superconducting. That is, an electrical current can be induced in them by an energizing power supply, and that current will persist for many years even after the power supply is removed. This essentially constant electrical current is used to generate high static magnetic fields that can be several times higher than those obtained with ferromagnetic-core magnets. Ensuring that the superconducting coil is immersed in liquid helium at all times is the only maintenance needed to sustain these high fields.

Figure 2 contains a diagram of a typical superconducting magnet. The superconducting solenoid is immersed in a liquid-helium Dewar at 4.2 K. This unit is itself contained in a liquid-nitrogen Dewar at 77.4 K. Each Dewar is surrounded by a vacuumed space and reflective film coating to prevent outside heat of the laboratory from entering the helium Dewar. The central core or room-temperature bore provides room for the stack that contains the shim coils for room-temperature shimming. Finally the probe is inserted into the stack. Samples are injected or ejected from the probe by means of a jet of filtered and dried air or nitrogen.

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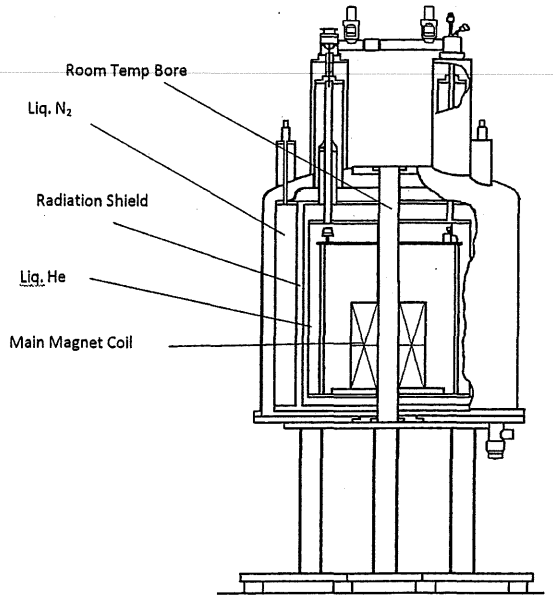

Figure 2. Schematic representation of a superconducting magnet.

An important recent advance in magnet technology has been the construction of shielded magnets with stray fields that can extend only one or two meters from the center of the magnet in three dimensions, thereby making magnet siting a far easier task than it was with unshielded magnets.

In addition to the main solenoid, the helium Dewar also contains several other superconducting coils that are used to shim the main magnetic field as a first step in attaining very high field homogeneity. Further shimming is accomplished by 20–30 shim coils in the room-temperature shim stack that is inserted in the bore of the magnet. These coils operate at ambient temperature and are used to generate small magnetic fields that oppose and cancel inhomogeneities caused by the surroundings, the probe, or the sample itself. Computer software has taken over a large amount of the tedious job of shimming the magnet homogeneity, a critical task for obtaining good NMR data. Using the lock signal from the sample, spectroscopists can generate a field map for each of the shim coils. Using this map, the computer then calculates the amount of current that should be applied to each of the shim coils to maximize the magnet homogeneity. Typically this operation takes less than a minute for shimming the on-axis (z_1 to z_6) shim coils. The off-axis (x/y) inhomogeneities can be compensated for in a similar manner but usually in a longer period of time (15–20 minutes) because of the larger number of off-axis coils.

The Probe

The NMR probe may be the most important part of the instrument. The probe consists of one or two rf coils. Each coil, which is inductive (L), is in a circuit that contains several tunable capacitive (C) elements. These elements are adjusted to enable the probe to transmit and receive at the Larmor frequency, ν_0. For a given nucleus, the probe tuning is determined by [$\nu = 1/(2\pi(LC)^{1/2})$]. A pulse of rf at the Larmor frequency results in an applied magnetic field (B_1). To induce a transition, B_1 must be applied perpendicular to the static field generated by the magnet superconducting coil. The rf coil not only transmits the excitation pulse but is also electronically switched to receive the rf signal from the sample.

The most common NMR sample tube is the 5-mm (od) NMR tube. However, probes have been designed in many forms. Some probes can accommodate 10- or 20-mm tubes for samples that are not in limited supply, such as petroleum and polymers. For limited amounts of precious samples, probes have been made to accept tubes as small as 1 mm, which can accommodate solutions as small as 5 µL. Also, flow probes are available to obtain data directly from a liquid chromatography effluent.

Probes are available in a large number of coil configurations. The most common probe usually contains a broadband observe coil that is tunable over a wide range of frequencies (^{31}P to ^{109}Ag), a decoupler (^{1}H) coil, and a coil tuned to deuterium (^{2}H) for field-frequency lock. Usually, the decoupler coil is double-tuned for both ^{1}H and ^{2}H. Probe configurations can include as many as four channels for multidimensional work with biological macromolecules.

The probe can come in a format where the X-nucleus observe-coil (e.g., ^{13}C) is wound closest to the sample for highest sensitivity, and the decoupler coil (^{1}H) is farther away. The inverse configuration is also available to provide the maximum signal-to-noise (S/N) ratio for ^{1}H, the detected nucleus in two-dimensional (2-D) indirect heteronuclear experiments, such as heteronuclear single-quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond correlation (HMBC) where the X-nucleus is indirectly detected via the ^{1}H frequency. Recent advances in probe technology have resulted in a single probe combining the sensitivity of the direct and inverse detection probes described above. Another advancement in probe design is the cryogenically cooled probe wherein the rf coils and their preamplifiers are held at close to liquid helium temperatures (20 K). Because rf electronics generate lower noise levels at colder temperatures, S/N ratios can be increased by at least a factor of four in these probes. Because the S/N ratio of a spectrum is given by $n^{1/2}$, where n is the number of acquisitions, an enhancement by a factor of four to the initial S/N ratio translates to a savings of 16 in time or to a four-fold reduction in sample size.

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Probes can also be equipped with gradient coils that can apply a magnetic field gradient in the *z* only or *x*, *y*, and *z* directions. These gradients are used to study diffusion or, more commonly, to be integral parts of pulse sequences because they provide an efficient means for selecting specific coherences in 2-D experiments.

In addition to the electronic coils, probes usually come equipped with a heater coil that enables variable temperature work from −100° to +150°. Probes also have gas outlets to allow sample insertion and ejection from the probe as well as sample spinning.

For solid-state samples the probes come with cylindrical rotors that are filled with the sample and capped. The entire rotor can then be oriented at an angle of 54.74° (known as the magic angle) relative to the magnetic field direction and spun at rates up to 70 kHz. Rapid rotation at this angle helps remove the chemical shift anisotropy and dipolar coupling so that the very broad resonances observed in the solid state can be reduced considerably.

The Console and Computer

The NMR console has the primary function of generating the various Larmor frequencies required for a given experiment, amplifying and transmitting these frequencies to the probe, and detecting the resulting signals that are transmitted from the probe so that they can be used to create an NMR spectrum. In addition to these primary functions, the console performs many more operations, all of which are computer-controlled. *Figure 3* contains a schematic diagram illustrating the various components of the current majority of spectrometer consoles.

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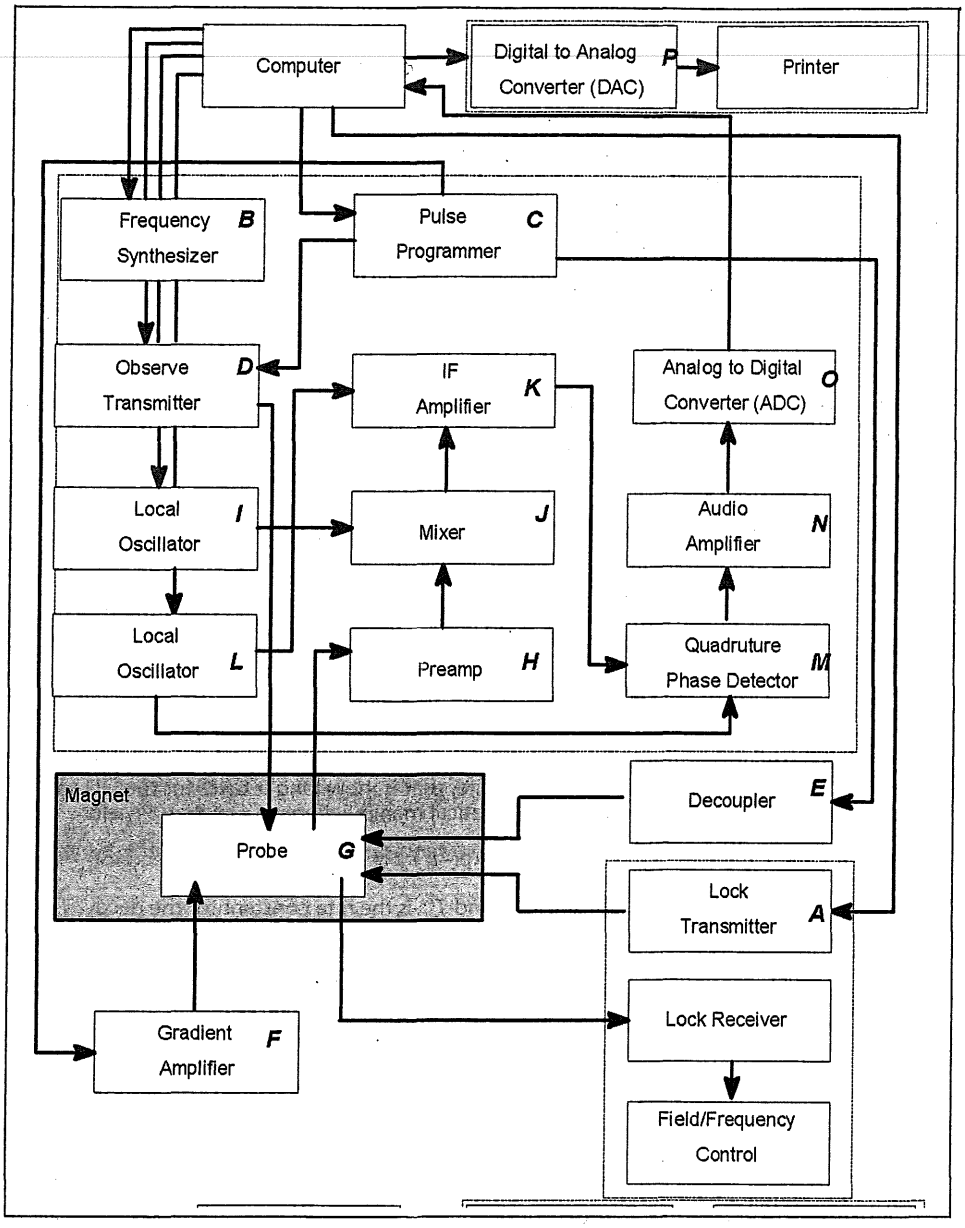

Figure 3. Schematic diagram of an NMR spectrometer.

The computer continually controls the signal to the lock transmitter (*A*) so the resonance of the lock material can be detected in the lock receiver to maintain field/frequency control. The computer also controls the frequency synthesizer (*B*) that generates the various frequencies that are close to the Larmor frequencies of the observed or perturbed nuclei. For a specific experiment, the computer triggers the pulse programmer (*C*), which, in turn, sends timing signals (pulse width, phase, and shape) to the observer transmitter (*D*), the decoupler (*E*), and the gradient amplifier (*F*), depending on which of these units are needed for that experiment. Once the observed nucleus in the probe (*G*) is excited by a pulse from the observed transmitter, the resultant rf signal is amplified in the preamplifier (*H*). Then the signal is mixed (*J*) with a local oscillator (*I*) to generate a lower rf frequency, called the intermediate frequency (IF) that is further amplified (*K*). A second mixing stage, this time with a different local oscillator (*L*), results in an audio signal that is then detected in quadrature (*M*) before being amplified (*N*), converted (*O*) from an analog to a digital signal, and stored in the computer. After the signal is processed to create a spectrum, the digital data can be converted back to an analog signal in a digital-to-analog converter (*P*) and printed.

The output from the phase-sensitive detector (*M*) is the free induction decay (FID), which is a time-domain signal, $f(t)$.

When two detectors are used (*M*) with their reference frequencies shifted from each other by 90°, frequencies that are positive with respect to the reference can be distinguished from those that are negative. This system is referred to as quadrature phase-sensitive detection (QPD). Each detector produces an FID, but they will always be 90° out of phase with respect to each

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other. One FID is called the real FID, and the other is called the imaginary FID. The Fourier transform that is performed in this case is called a complex Fourier transform. The combination of the real and imaginary FIDs yields the frequency spectrum, $F(\omega)$.

$$F(\omega) = \int_{-\infty}^{+\infty} f(t)\exp^{-i\omega t} dt \quad [8]$$

In addition to the Fourier transform process, the computer is also used for post-acquisition processing of the data. The frequency-domain spectrum that results from the complex Fourier transform can then be phased, baseline corrected to remove distortions, integrated to obtain peak areas, and peak picked to provide chemical shift information. The computer is also capable of providing spectra calculated from chemical shift and coupling values, curve-fitting resonances, and deconvoluting complex overlapped peaks. Finally, the digital data can be converted to their analog form by a DAC (***O***) and printed.

RELAXATION

NMR includes two types of relaxation: *Spin-Spin Relaxation*, sometimes referred to as *Transverse Relaxation*, or T_2 Relaxation, and *Spin-Lattice Relaxation*, sometimes referred to as *Longitudinal Relaxation*, or T_1 Relaxation. At least two mechanisms contribute to *Transverse Relaxation*: loss of signal due to B_0 inhomogeneity and the natural relaxation that would take place even in a perfectly homogeneous field. The combined effects of these two mechanisms produce a new time constant for the relaxation, which is referred to as T_2*.

Spin-Spin Relaxation (Transverse Relaxation)

After an rf pulse, the component of M_0 in the (x,y) plane, M_{xy}, will gradually decay toward zero. The process is first order, and as it is in other types of first-order processes, the instantaneous rate of decay of M_{xy} is directly proportional to its displacement from equilibrium. The further M_{xy} is displaced from zero, the faster it decays, and as it approaches zero it decays more and more slowly. Hence, *Equation 9* applies.

$$dM_{xy}/dt \propto (-M_{xy}) \quad [9]$$

The process is analogous to the decay of a radioactive element. However, spectroscopists do not speak of the rate of decay of an FID in terms of its half-life. Instead, they speak of its 1/e-life, that is, how long it takes for the FID to decay to a value that equals 1/e of its original value at time zero. Standard mathematical manipulations of *Equation 9* yield

$$M_{xy} = M_0\exp(-t/T_2^*) \quad [10]$$

where M_0 is the equilibrium distribution given by *Equation 9*, and T_2* is the rate constant for the decay. T_2* is a *measure* of how fast the signal decays, not how long it takes to decay. The rate of decay is fastest immediately after the pulse because then it is furthest from its equilibrium position, zero. If the rate remained constant at this initial rate, then the signal would be completely decayed after one T_2*.

Table 3 was prepared from *Equation 10*. It shows the percentage of M_{xy} remaining as a function of time after a 90° pulse. In *Table 3* time is given as the number of T_2*s.

Table 3. Percent of M_{xy} Remaining as a Function of Time, in Units of T_2*

Time/T_2*	0.0	0.5	1.0	1.5	2.0	3.0	4.0	5.0
% Remaining	100.0	60.7	36.8	22.3	13.5	5.0	1.8	0.7

M_{xy} asymptotically approaches zero, and it would take infinitely long for complete decay, but the decay is normally considered to be complete when the time has reached 3 to 5 times T_2*. Hence, these times are commonly used as acquisition times. If acquisition times that are shorter than 3 times T_2* are used, the FID is truncated and subsequent Fourier transform leads to visible baseline artifacts.

The decay of the FID produces the peak width in the final spectrum. The faster the decay, the broader is the line. The mathematical relationship is:

$$\Delta\nu_{1/2} = 1/(\pi T_2^*) \quad [11]$$

where $\Delta\nu_{1/2}$ is the width of the peak at its half-height.

Spin-Lattice Relaxation (Longitudinal Relaxation)

After an rf pulse, nuclei are excited from the low-energy state into the high-energy state. The nuclei will eventually relax back to establish the Boltzmann distribution (see *Equation 5*), and this process is called spin-lattice relaxation. The recovery process is first-order, and as it is in other types of first-order processes, the instantaneous rate of growth of M_z is directly proportional to its displacement from equilibrium. The farther M_z is displaced from M_0, the faster it grows back, and as it approaches M_0 it grows back more and more slowly. Hence, *Equation 12* applies.

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$$dM_z/dt \propto (M_0 - M_z) \text{ [12]}$$

Standard mathematical manipulations yield

$$M_z = M_0(1 - \exp(-t/T_1) \text{ [13]}$$

Note that T_1 is a measure of how fast M_z grows back to M_0—it is not how long it takes to grow back. *Table 4* was prepared from *Equation 13* and shows the percentage of recovery of M_z as a function of time after a 90° pulse. In this table, time is given as the number of T_1s.

Table 4. Percent Recovery of M_z as a Function of Time, in Units of T_1

Time/T_1	0.0	0.5	1.0	1.5	2.0	3.0	4.0	5.0
% Recovery	0.0	39.3	63.2	77.7	86.5	95	98.2	99.3

As M_z asymptotically approaches M_0, it takes infinitely long for 100% recovery. However, recovery is normally considered to be complete when it has reached 99%. Hence, relaxation delays of $5T_1$ are commonly used in pulse sequences. The rate of return to M_0 is fastest immediately after the pulse because that is when M_0 is farthest from its equilibrium position. If the rate remained constant at this initial rate, then full recovery would be achieved after one T_1.

TIP ANGLE

During an rf pulse, a magnetic field (B_1) is applied to the sample. The magnetization vector M precesses about B_1 according to

$$\omega_1 = \gamma B_1 \text{ [14]}$$

where ω_1 is the precessional frequency and B_1 is the strength of the magnetic field applied to the sample. During the time that the pulse is applied, M precesses to an angle (α) given by the precessional rate multiplied by the width of the pulse, in time, (PW). Then,

$$PW \times \omega_1 = \alpha = PW \times \gamma B_1 \text{ [15]}$$

Tip angles typically are expressed in units of degrees or in radians. For example, a 90° pulse is sometimes referred to as a $\pi/2$ pulse.

Optimum Tip Angle or Ernst Angle

Time averaging to improve the S/N ratio is accomplished by a delay-pulse-acquire sequence that is repeated as necessary. Suppose that during the first pulse, M_0 precesses 30° about B_1 before the pulse is turned off. At this time the magnitude of M_z is $M_0\cos 30°$. At the end of the pulse, M_z will begin to grow back toward its equilibrium value, M_0. Typically, the second pulse is applied before M_z reaches M_0, driving M_z down even farther away from M_0. Consequently, M_z begins to grow back faster because it is even farther away from its equilibrium value. After 6–10 pulses, M_z will grow back an amount that is equal to the incremental displacement caused by each succeeding pulse and will reach a new equilibrium or steady state. Each succeeding pulse continues to tip this new steady-state value of M_z by 30°, resulting in signal intensity for each pulse.

The position of the steady-state equilibrium is determined by three factors: the T_1, the tip angle, and the time between pulses. On the one hand, for a given T_1 and time between pulses, if the tip angle is too large then the steady-state value of M_z will be close to the origin, affording only a small signal. On the other hand, if the tip angle is too small, e.g., 5°, then the steady-state value will be large, but with a small angle the value of $M_z\sin 5°$ will also be small, again affording a small signal. The optimum angle is frequently called the Ernst angle, which is given by:

$$\cos \alpha_{opt} = \exp(-PR/T_1) \text{ [16]}$$

$$\alpha_{opt} = \text{arc cos}[\exp(-PR/T_1)] \text{ [17]}$$

where PR is the time between pulses, or pulse-repetition time. This time is the sum of the acquisition time used to collect the FID plus any relaxation delay used. This angle provides a reasonably large steady-state value combined with a reasonably large angle and will produce the best S/N ratio per unit time. The value of T_1 to be used in *Equation 17* should be for the longest relaxing nucleus in the molecule.

RELAXATION DELAY

Surprisingly, the optimum S/N ratio per unit time is obtained when no relaxation delay is used for a given T_1. That is when PR equals to AT, the acquisition time. However, because the nuclei in most molecules do not have the same T_1 values, the relative intensity relationship will be lost.

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For typical quantitation experiments, a relaxation delay is used and should be at least five times the longest T_1 expected for any of the nuclei in the molecule; and the pulse width should be set to 90°. Further details are provided in the section *Quantitative Applications*.

RESOLUTION

In NMR spectroscopy the typical definition of resolution is the ability to distinguish between two closely spaced resonance peaks in a spectrum. The industry standard for measuring resolution is to measure the width of a single peak, in units of Hz, at the half-height of the peak.

The uncertainty principle determines the best resolution that can be achieved in an NMR spectrum. The maximum resolution possible, or the minimum separation that can be observed between two frequencies, $\Delta\nu$, in a spectrum is equal to the reciprocal of the acquisition time, AT, of the FID.

$$\Delta\nu = 1/\Delta t = 1/AT \quad [18]$$

The time set by the spectrometer operator should not exceed the required AT, because this would result in the collection of only noise after the signal has decayed to near zero. Collecting this noise does not improve the resolution.

POSTACQUISITION DATA PROCESSING

The final appearance of the spectrum can usually be improved by applying a variety of mathematical procedures to the FID before the Fourier transform is performed. The two most common procedures are multiplying the FID by a mathematical function, generally known as a *window* function; or appending zeros to the end of the FID, generally known as *zero filling*.

Window Functions

Two types are generally used: one for increasing the resolution and another for increasing the S/N ratio.

INCREASING THE RESOLUTION

The decay of the signal produces a peak width in the spectrum, and if this decay can be removed, then the resonance peak would consist of a single point, i.e., an infinitely narrow peak. The decay of the signal can be represented by $\exp(-t/T_2^*)$. Hence the full equation representing the decaying signal is

$$A(t) = A_0\exp(-t/T_2^*)\cos(\omega t + \theta) \quad [19]$$

If the FID is multiplied by an increasing function that exactly cancels the decay, then the peak width will be removed. This can be achieved by multiplying the FID by $\exp(t/T_2^*)$. Then *Equation 19* becomes

$$A(t) = A_0\exp(0)\cos(\omega t + \theta) = A_0\cos(\omega t + \theta) \quad [20]$$

Unfortunately, the application of this function as described above will also disproportionately increase the noise power at the tail of the FID. The S/N ratio in the final spectrum is so poor that this function is not used without modifications. Typically, the beginning of the FID, where the S/N ratio is better, is multiplied up but then the tail of the FID, where S/N ratio is poorer, is multiplied down. Two commonly used functions that accomplish this are the Gaussian function and the transform of reversed-added FIDs (TRAF) function for resolution. The latter is sometimes given the name TRAFR by instrument manufacturers. It affords the same resolution enhancement as the Gaussian function but with much less degradation in overall S/N ratio. Numerous other window functions have been proposed though not always widely used. It should also be noted that in the quantitative experiments increasing the resolution should be used with caution because it may change the accuracy of signal integration in the spectrum.

INCREASING THE S/N RATIO

The overall S/N ratio in the spectrum can be increased by weighting the points at the beginning of the FID more highly than at the tail. This is because the S/N ratio is the highest in the beginning and the lowest at the tail. The weighting is often accomplished by multiplying the raw FID by a function that decreases with time. A popular function that gives the greatest increase in the S/N ratio is called the *matched filter*. It weights each point in the FID by an amount proportional to the S/N ratio at that point. A function that accomplishes this must match the decay. Hence, the FID is multiplied by $\exp(-t/T_2^*)$.

The penalty for the use of the matched filter is a loss of resolution that equals a doubling of the peak width. When the original decay is multiplied by the matched filter, then the new decay is given by the following:

$$\exp(-t/T_2^*) \times \exp(-t/T_2^*) = \exp(-2t/T2^*) = \exp(-t/0.5T_2^*) \quad [21]$$

The FID then appears to have decayed with a T_2^* equal to one-half of the original, and according to *Equation 11* and *18* the peak width will double. Multiplying the FID by a steeper decay in an attempt to weight the beginning points eore results only in less of an increase in S/N ratio and a greater increase in peak width.

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Another function that accomplishes the same increase in S/N ratio, but without any change in resolution, is the TRAF function for sensitivity, which is sometimes given the name TRAFS by instrument manufacturers.

Zero Filling

Spectroscopists can improve the overall appearance of the spectrum considerably by appending zeros to the end of the FID before the Fourier transform is performed. This process results in placing more points on every resonance peak in the spectrum. The most common procedure is to append a number of zeros equal to the number used to collect the FID. Adding more zeros will result in only a very slight further improvement.

Although all of the peaks are better defined with zero filling, the resolution is not increased. For example, in a case where the separation between two lines is closer than the peak widths, only a single broadened line will result. Zero filling will not resolve these peaks—it will only place more points on an already broadened line. Only an increase in the acquisition time of the signal or the use of a window function could resolve the peaks.

Zero filling has a beneficial effect on quantitation. Integration of a digital spectrum is accomplished by taking the intensity at a given point and adding to it the intensity at successive points. If the number of data points is insufficient to depict the actual peak shape, the resultant integral of that peak will not be accurately determined. Therefore, zero filling until each peak is represented by at least 7–10 data points results in a more accurate integration. To obtain reliable peak representation and quantitative peak integration there should be at least 4–5 data points above the full width at half height of a peak.

GENERAL PROCEDURE FOR STRUCTURE IDENTIFICATION

NMR spectroscopy is a powerful technique for structure identification because of its specificity of detecting certain nuclei such as ^{1}H, ^{13}C, ^{31}P, and ^{19}F. Typically, a routine identification test can be performed by ^{1}H NMR spectroscopy in a short period of time for simple molecules. The basis for identification is provided by a comparison of the signals from the test sample with the expected signals from a qualified reference standard. A positive identification can be concluded when the chemical shifts, multiplicities, and coupling constants of the spectrum of the test sample match those of the reference standard or, in the case of a *USP* monograph, the values listed in the monograph.

Data may be made unacceptable for analysis if incorrect sample preparation or poor adjustment of spectrometer parameters leads to poor resolution, decreased sensitivity, and spectral artifacts. It is preferable that the operator be familiar with the basic theory of NMR and operation of the spectrometer. Frequent checks of instrument performance are essential.

The procedures discussed here refer specifically to ^{1}H and ^{13}C NMR, but they are applicable, with modification, to other nuclei. The discussion assumes that the NMR spectra are obtained from solutions in suitable solvents.

Selection of Solvent

Deuterated solvents are usually used to prepare solutions for NMR analysis because they are readily available, have greatly reduced ^{1}H signals from solvents in ^{1}H spectra, and have the added advantage of providing a lock signal. Select a solvent whose residual ^{1}H signals will not interfere with signals of the analyte. If a residual ^{1}H solvent peak might interfere with any signals from the sample solution and another solvent is not possible, then the ^{2}H isotopic purity of the solvent should be as high as possible. Some solvents (e.g., D_2O or CD_3OD) have labile protons that can enter into fast exchange reactions with the labile protons in the analyte. This may eliminate resonance signals from –COOH, –OH, and $–NH_2$ structural groups. The most commonly used solvents for proton and carbon NMR are listed in *Table 5* along with their residual ^{1}H and ^{13}C chemical shifts as well as the multiplicities of these resonances caused by coupling to deuterium.

Table 5. Solvents Commonly Used for $^{1}H/^{13}C$ NMR Chemical Shifts

Solvent	Residual $^{1}H/^{13}C$ Signal ($\delta^{a,b}$) and Multiplicity	
	^{1}H	^{13}C
$CDCl_3$	7.27	77.23 (3)
CD_3OD	3.35, 4.78	49.15 (7)
$(CD_3)_2CO$	2.05	206.68 (1) 29.92 (7)
D_2O	4.7[c]	—
$(CD_3)_2SO$	2.50	39.51 (7)
C_6D_6	7.20	128.39 (3)
Dioxane-d_8	3.55	66.66 (5)
CD_3CO_2D	2.05, 11.65[c]	178.99 (1) 20.0 (7)

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Table 5. Solvents Commonly Used for $^{1}H/^{13}C$ NMR Chemical Shifts *(continued)*

Solvent	Residual $^{1}H/^{13}C$ Signal ($\delta^{a,b}$) and Multiplicity	
$(CD_3)_2NCDO$	2.77, 2.93, 8.05	163.15 (3) 34.89 (7) 29.76 (7)

[a] Chemical shifts were measured at 295 K>.
[b] δ in ppm relative to TMS at 0 ppm.
[c] Labile hydrogen.

Sample Preparation

For *USP* procedures, directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment. Typically, NMR sample solutions are prepared so that they contain from a few to 50 mg/mL. Detection of minor contaminants may require higher concentrations. In some cases such as polymers, even higher concentrations can be used. The solutions are prepared in separate vials and are transferred to the NMR tube. The volume required depends on the size of the NMR tube and on the geometry of the probe. The level of the solution in the tube must be high enough to extend beyond the coils when the tube is inserted in the instrument probe.

The NMR tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5- or 10-mm outside diameter (OD) and a length between 15 and 20 cm, but 1- and 3-mm (OD) NMR tubes are becoming more common, and tubes as large as 20 mm (OD) have been used.

Procedure

The NMR tube is placed in a probe located in the magnetic field. Although samples traditionally have been spun to average the nonradial field gradients, the quality of the shim coils no longer makes spinning a requirement, and, in the case of many 2-D experiments, the sample should not be spun. The magnetic field's homogeneity is optimized by shimming, a function that is largely being taken over by the computer in most modern spectrometers. Probe tuning is optimized for the frequency being observed and is matched to the impedance of the spectrometer.

The computer serves to control all operations of the spectrometer from running the pulse program to storing and processing the data. The experimental setup involves selecting values for a large number of variables, including the spectral width to be examined, the duration of the excitation pulse (*PW*), the time interval over which data will be acquired (*AT*), the number of transients to be accumulated, and the delay between one acquisition and the next (relaxation delay). The acquisition time for one transient is on the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment and can vary from a few for most ^{1}H experiments to several thousand for ^{13}C spectra. At the end of the experiment, the signal (FID) is stored in digitized form in the computer memory and may be displayed on the monitor. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum, which can be further analyzed to obtain peak positions (chemical shifts) and intensities.

Structure Elucidation by NMR

The simplest case of using NMR spectroscopy to elucidate an unknown structure is to obtain a match with a spectrum from a reference standard or from a database. The informational content of an NMR spectrum is sufficient for deducing structures of organic molecules even when qualified reference standards or spectra are not available. Relatively simple structures can be identified using chemical shifts, coupling patterns, and intensities obtained from one-dimensional (1-D) ^{1}H and ^{13}C spectra. For more complex structures, spectroscopists may have to obtain two-dimensional (2-D) spectra from experiments that have been developed to determine homo- or heteronuclear connectivities.

Two-dimensional spectra are characterized by two frequency axes. The intensity, which is mathematically another dimension, is not considered to be a dimension in 2-D NMR because it is not an axis that spreads out the chemical shift. All modern 2-D experiments consist of at least two pulses separated by a time period, labeled t_1, the evolution period, and a period of time used for collecting the signals, labeled t_2, the detection period. A COrrelation SpectroscopY (COSY) sequence is the simplest of all and is shown in *Figure 4*.

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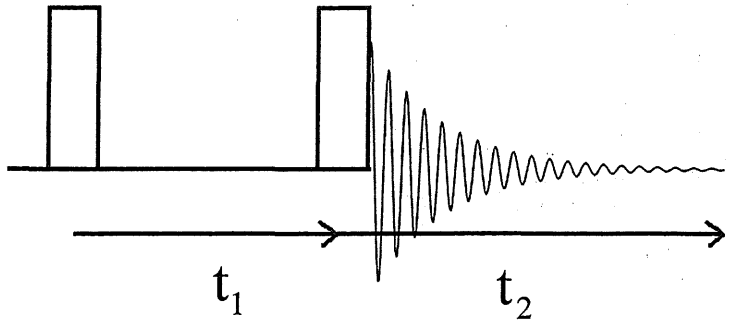

Figure 4. Pulse sequence for a COSY experiment.

A series of such sequences is performed using an incremental increase in the evolution period. Fourier transforms of the FIDs produced during each of the t_2 detection periods are stacked, a second Fourier transform is performed along this t_1 new axis, resulting in a plot of amplitude along two frequency axes, F_2 and F_1.

The next simplest addition to the COSY sequence is another pulse. The Nuclear Overhauser Effect SpectroscopY (NOESY) sequence is an example and is shown in *Figure 5*.

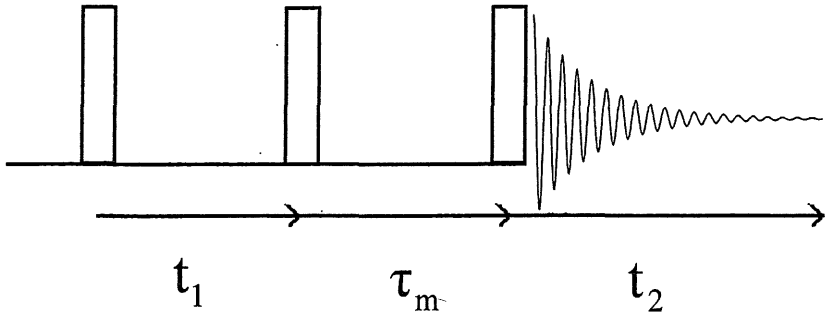

Figure 5. Pulse sequence for a NOESY experiment.

The evolution period is again the time between the first two pulses and is incremented as in the COSY experiment. The time between the second and third pulses is fixed and is not incremented. This period of time is labeled τ_m and is called the mixing time. Cross-polarization occurs during this period.

The COSY and NOESY sequences described above are the two simplest 2-D experiments to perform. Many other experiments have been developed and contain a very complicated series of pulses with different pulse widths, time delays, gated decoupling, and pulsed field gradients. Some of these experiments are described below.

Strategies for Establishment of Homonuclear Connectivities

Assigning signals based on chemical shifts, spin multiplicity, and coupling constants serves as a starting point for structural elucidation. Structure elucidation is simplified if one can establish molecular connectivity between homonuclear spins. This can be done using correlations via bonds (scalar coupling, sometimes referred to as *J* coupling) or via spatial (dipolar coupling) interactions. This section describes NMR techniques that can be used for the study of homonuclear connectivity. Popular experiments in this category are COSY, total correlated spectroscopy (TOCSY), NOESY, and rotating frame Overhauser effect spectroscopy (ROESY).

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COSY

COSY has become a routine 2-D ^{1}H NMR experiment that can quickly provide the proton–proton connectivity for spin systems connected by two or three chemical bonds. In a COSY spectrum, the contour plot typically shows diagonal and off-diagonal cross-peaks. The diagonal peaks correspond to the places in 2-D space where chemical shifts of the same nucleus in the 1-D ^{1}H NMR spectrum intersect. The off-diagonal cross-peaks occur in 2-D space where the chemical shift of one nucleus intersects the chemical shift of a different nucleus to which it is coupled. The COSY spectrum enables the identification of scalar-coupled spins that are in geminal or vicinal positions. Generally, one starts the assignment process by selecting a resonance in the COSY spectrum that has already been identified in the 1-D ^{1}H NMR spectrum. Then, the off-diagonal peaks between this resonance and any others determine the resonances of the neighboring protons to which it is coupled. The neighboring protons identified in this way then serve as the next points to examine for cross-peaks to other protons, and so forth. The process continues until all coupled spin systems are identified.

Thus, a COSY experiment can provide useful information about proton–proton connectivity for various fragments of the molecules under examination. The relationship between these fragments may be difficult to establish because of an interruption of the coupling between the two fragments. For instance, if two segments are connected through quaternary carbons or heteronuclei, the very small four-bond coupling between two protons on the two separate fragments usually cannot be detected by COSY. Basic COSY experiments are usually processed using magnitude mode, which results in broad bases to the peaks. The diagonal peaks may be so broad that spin systems with close chemical shifts may not be observable because the broadness of the diagonal peaks will cause them to overlap the off-diagonal peaks. An example of a COSY experiment, specifically a gradient, or gCOSY, is shown in *Figure 6*.

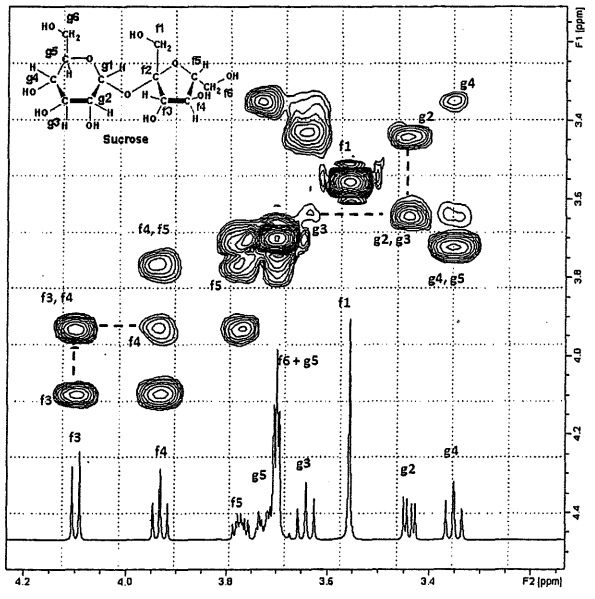

Figure 6. Partial COSY spectrum of sucrose. Dotted lines show how two off-diagonal (f3, f4 and g2, g3) contours are used to determine which nuclei are coupled to each other.

A variety of COSY experiments have been devised to improve upon the original experiment. Perhaps the most important change has been to use gradients to acquire a gCOSY spectrum. The classic COSY experiment uses a 90° pulse to generate transverse magnetization and relies on elaborate phase cycling to cancel unwanted signals over many scans, resulting in long experiment times. In a gCOSY experiment, which requires a probe capable of a pulsed field gradient (PFG), a magnetic field gradient pulse dephases any coherent magnetization in the *xy* plane. If a second gradient pulse of the proper strength is applied in the opposite direction, it will cause any dephased double-quantum magnetization to refocus. Hence, only those signals will be received. The strengths of the gradients can be used to select single-, double- or triple-quantum coherences.

The gradient pulses in a gCOSY experiment can be used to prevent the refocusing of magnetizations that cause the artifacts in the classic COSY experiment. Hence, instead of requiring a minimum of eight phase-cycled acquisitions for each data increment in the second dimension, a gCOSY spectrum requires only a single acquisition per increment. For samples that are sufficiently concentrated to produce an acceptable S/N in only one acquisition, this greatly shortens the experimental time.

Phase-sensitive COSY experiments have been developed to overcome the problem of overlap with closely spaced chemical shifts. A phase-sensitive COSY results in pure absorptive peaks with narrower peaks than are generated by the classic-magnitude COSY. These narrower peaks allow better resolution of resonances that are close to diagonal peaks.

Double-quantum filtered COSY (DQF-COSY) was developed to overcome the problem caused by intense signals from functional groups such as methyls. The singlets from these groups do not provide useful connectivity information, but their intensity often limits the dynamic range of the experiment, making it difficult to observe other weaker signals. The pulse sequences of DQF-COSY detect the spin systems that have only double-quantum transitions. Isolated singlets are not selected

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and thus are filtered out of the final 2-D spectrum. In addition, a reduction in the overall intensity of the diagonal signals is achieved with an increase in the intensities of off-diagonal signals.

TOCSY (OR HOHAHA—HOMONUCLEAR HARTMANN HAHN)

The ^{1}H–^{1}H TOCSY experiment is closely related to COSY but differs because it yields correlations for every spin in a coupled network. This is especially useful when multiplets overlap or there is extensive strong coupling. For example, consider the network –CHa–CHb–CHc–CHd–CHe–, where each CHn stands for a spin that is coupled through three bonds to the adjacent spin. A COSY spectrum would show correlations for each adjacent pair of hydrogens. On the one hand, the Hb resonance would show connectivities to Ha and Hc but not to Hd. A partial correlation is revealed for each CHn. On the other hand, a TOCSY spectrum would show all off-diagonal contours for every spin in this network. That is, for every peak in this coupling network there would be off-diagonal contours corresponding to CHa, CHb, CHc, CHd, and CHe. Thus, a TOSCY spectrum, such as the one shown in *Figure 7*, affirmatively identifies all of the spins within the same coupling network. This pattern is easily recognized, especially when there is extensive overlap with other coupled networks. However, a TOSCY experiment cannot establish connectivity between separate networks that are interrupted by heteronuclear atoms, quaternary carbons, or a carbon bearing only exchangeable protons. A TOCSY experiment is useful for the study of large molecules with many separated coupling networks such as peptides, proteins, oligosaccharides, and polysaccharides.

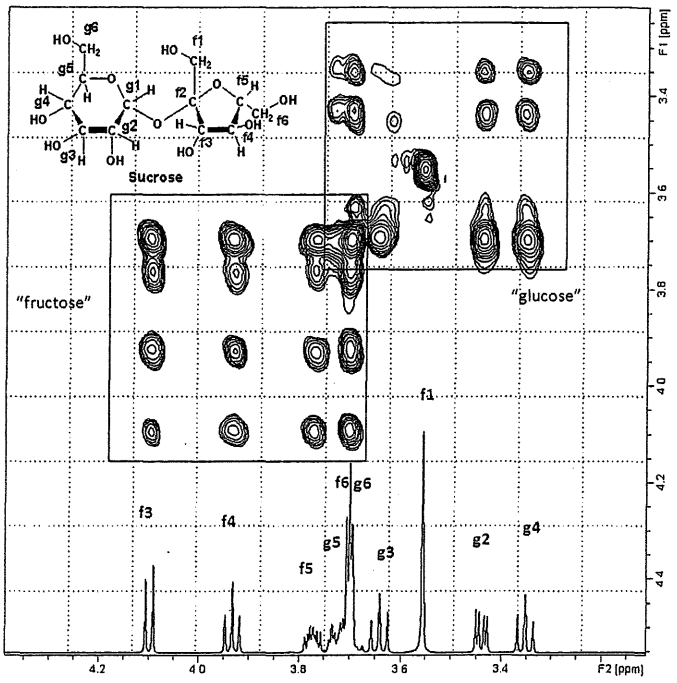

Figure 7. Partial TOCSY spectrum of sucrose showing two sets of contours corresponding to all the coupled nuclei in the glucose and fructose rings in sucrose. The contour for the f1 resonance falls within the glucose spin system and has no coupling.

NOESY

The NOESY experiment gives correlations between protons that are close to each other in space even though they may not be connected by bonds. These through-space correlations are made via spin-lattice relaxation. Dipole interactions between protons close in space generate NOE transfers, and the magnetization is aligned along the z axis (B_0), producing positive or negative intensity changes that yield cross-peaks that are not normally observable in a COSY spectrum. The sign of the NOESY peaks depends on the size and mobility of the molecule under study. Used in combination with other techniques, a NOESY experiment can establish spatial relations for particular spins and can provide critical information about ring structures and conformations.

^{1}H–^{1}H ROESY

The ROESY experiment is similar to a NOESY experiment insofar as it also provides correlations between protons that are close to each other in space, whether or not they are connected via bonds. A ROESY spectrum yields through-space correlations via spin-spin relaxation in the rotating frame. The ROESY experiment utilizes a spin–lock sequence as a mixing time during which NOE transfer occurs among all components of the spins locked in the xy plane. In contrast to a NOESY experiment, in which NOE transfer occurs while magnetizations are aligned along the z axis (B_0) producing positive or negative intensity changes, the ROESY experiment depends on NOE transfers occurring in the rotating frame under the influence of a B_1 magnetic field. This always results in positive signals no matter how large the molecule or whether its motion is fast or slow. Therefore, a

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ROESY experiment frequently will provide through-space correlations when the same correlations in a NOESY experiment cannot be detected because of molecule mobility.

INCREDIBLE NATURAL ABUNDANCE DOUBLE QUANTUM TRANSFER EXPERIMENT

The Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE) uses a double quantum coherence to provide information about ^{13}C nuclei directly coupled to other ^{13}C nuclei. Thus it provides the same sort of information that is available using a COSY experiment for proton couplings. Because of the low probability of two ^{13}C nuclei being attached to each other (only 1 in 10,000 molecules), this technique is usually one of last resort. The ever-increasing sensitivity of cryogenically cooled probes used in modern instrumentation makes this experiment practical in some cases.

Strategies for Establishment of Heteronuclear Connectivities

Although homonuclear ^{1}H–^{1}H connectivity is one important aspect of structure elucidation of organic molecules, the establishment of heteronuclear connectivities is equally important, although somewhat more difficult to obtain given the lower abundance of most heteroatoms. If one has partial assignments in either the ^{1}H or ^{13}C spectrum, the knowledge of this connectivity leads to a much fuller assignment of both spectra. Heteronuclear 2-D spectra do not exhibit a diagonal as is seen in homonuclear correlations. Rather, cross-peaks occur at the point of intersection of the ^{1}H and ^{13}C chemical shifts in the 2-D space as shown in *Figure 8*.

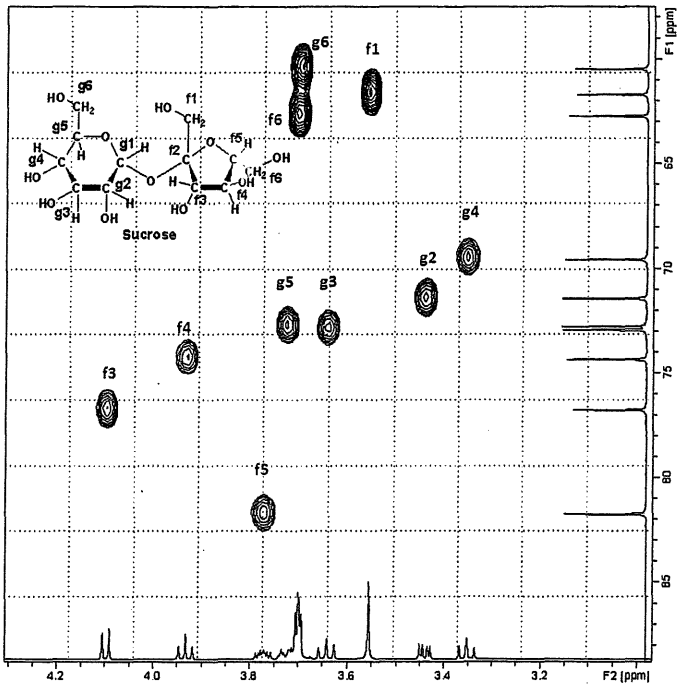

Figure 8. Partial $^{1}H/^{13}C$ HSQC spectrum of sucrose showing contours that indicate the one-bond correlation between a given hydrogen and the carbon to which it is attached.

Heteronuclear 2-D spectra are designed so that ^{1}H is the detected nucleus and are usually acquired using inverse- detection probes because the ^{1}H coil is wound closer to the sample than the broadband coil. This results in a better filling factor and a greater sensitivity for the ^{1}H coil. It should be noted that a newer configuration of coils has been developed that provides the same sensitivity but does not utilize the inverse-detection coil arrangement. Typical heteronuclear 2-D experiments include Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Quantum Coherence (HMQC), and Heteronuclear Multiple Bond Correlation (HMBC) experiments.

HMQC

The 2-D HMQC experiment provides information about correlation between protons and their attached heteronuclei via the heteronuclear scalar coupling. The sequence selects double quantum coherence transfer between scalar-coupled spins (^{13}C–^{1}H).

HSQC

HSQC spectroscopy is also an inverse chemical shift correlation experiment that yields the same information as HMQC, i.e., the identification of directly bonded hydrogen–carbon interactions. The correlation between heteronuclei is detected via the

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selection of single quantum coherence transfer using the insensitive nuclear enhancement by polarization transfer (INEPT) sequences. The main advantage of using this sequence instead of the HMQC sequence is that the F_1 domain does not contain any proton–proton couplings. Hence, the resolution is improved.

An interesting modification of this sequence is an edited HSQC experiment. This is a phase-sensitive experiment that not only gives one-bond correlations between hydrogen and carbon but also gives methyl and methine correlation peaks that are 180° out of phase with methylene resonances.

HMBC

HMBC spectroscopy is a modified version of HSQC and is suitable for determining long-range (> 1-bond) ^{1}H–^{13}C connectivity. Long-range heteronuclear correlation spectroscopy can yield signals for those nuclei that are separated by 2–4 bonds. This experiment, in conjunction with the other 2-D experiments discussed above, allows one to define the structure of a molecule in great detail.

QUANTITATIVE APPLICATIONS

NMR is one of the most useful techniques for quantitative analysis in chemistry. If appropriate experimental conditions are chosen, the relative intensities of resonances are proportional to the population of the nuclei causing those resonances. NMR experiments can be designed for relative or absolute quantitation, either with an internal standard or without one.

Experimental Design for Quantitative NMR

Design for quantitation involves the elimination or precise measurement of differences in intensities due to spin-lattice relaxation and NOE. The spin-lattice relaxation time (T_1) for all resonances used in the procedure can be measured with an inversion recovery pulse sequence. If a 90° pulse is used for excitation, quantitation at the level of 99.3% may be achieved with a recycle time, T_r (the sum of the relaxation delay and aquisition time), of $5 \times T_1$, and improved to even higher levels by using longer recycle times or shorter pulses. The general equation for the degree of quantitation, Q, of a resonance as a function of the pulse angle, α, and T_r, and T_1 is given by:

$$Q = \frac{1 - e^{-Tr/T1}}{1 - [e^{-Tr/T1} \cdot \cos(\alpha)]} \qquad [22]$$

If a 45° excitation pulse is used, with a $T_r = 5T_1$, $Q = 0.998$. However, the accuracy of quantitation in the final spectrum depends not only on the Q values of the resonances but also on the accuracy of the integration method and on the S/N ratio in the spectrum. Hence, Q values somewhat less than unity may be warranted and other angles and recycle times should be used.

The minimization of systematic quantitation bias should be sufficient for the intended use of the procedure. Alternatively, quantitative procedures may be developed using conditions for which Q is not unity for some or all resonances, provided the value of Q is precisely known and corrected for.

For quantitative methods using heteronuclei, the possibility of differential NOEs should be avoided by using a T_r at least 5 times the longest T_1 value and by using inverse gated decoupling (decoupler gated on only during the acquisition time). Preferably, 90° pulses should be used. For quantitative methods, relaxation agents are often used for shortening the T_1 values of heteronuclei.

The reproducibility of an NMR method depends on a variety of acquisition and processing parameters, all of which should be described in the procedure. These include pulse angle, acquisition time, relaxation delay, spectral width, number of points in the FID, number of acquisitions, number of points used (if any) for zero filling, line broadening, baseline correction, integral breaks, and temperature. For best reproducibility, integral breaks should be specified to 0.01 ppm for ^{1}H NMR methods and to 0.1 ppm for ^{13}C methods.

Quantitative analysis, as well as detection of trace impurities, has markedly improved with modern instrumentation. Stronger magnetic fields and improved probe technology have enhanced the sensitivity of NMR procedures in recent years.

SOLID-STATE NMR

The analytical usefulness of solid-state NMR spectroscopy for studying solid materials lies in the fact that the same types of nuclei in different *solid-state* environments exhibit different resonance frequencies. Applications of this technique in pharmaceutical analysis range from solid form (polymorph, solvate) identification and quantitation in bulk drug substances to physical/chemical profiling of dosage forms. The technique has the unique ability to probe electronic environments of specific nuclei in the solid state over a large timescale without the requirement of single-crystal substrates or even homogeneous samples. Methods and procedures presented herein are directed at observing ^{13}C, the most popular NMR nucleus for solids. The concepts may be equally applied to other relevant spin-1/2 nuclei such as low-natural-abundance ^{15}N as well as high-natural-abundance ^{31}P.

Cross-Polarization Magic Angle Spinning (CPMAS) NMR Technique

The basic principles of NMR are the same for solution and solid-state measurements, but conventional solution-phase 1-D NMR data acquisition techniques do not normally produce detectable spectra for solid samples because of low sensitivity and

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extensive line broadening. The sensitivity of the solid-state experiment is low for ^{13}C based on its 1.1% natural abundance and long spin-lattice (T_1) relaxation times. Line broadening arises primarily from dipole–dipole interactions and chemical shift anisotropy (CSA), which are not averaged to zero because of the fixed orientation of molecules in a packed solid sample vs the rapid molecular tumbling of the molecules when they are in solution. If not averaged, CSA results in the simultaneous observation of all different orientations of molecules with respect to the applied magnetic field. CSA patterns may span the width of an entire liquid spectrum. Three modifications to standard solution methods—cross-polarization (CP), magic angle spinning (MAS), and high-power ^1H decoupling—are routinely used in combination to obtain high-resolution solid-state NMR spectra.

CROSS-POLARIZATION (CP)

CP addresses the low sensitivity associated with collecting NMR spectra of dilute spin-1/2 nuclei such as ^{13}C. CP is a double-resonance procedure wherein abundant ^1H and rare ^{13}C spins are brought into resonance by simultaneously applying two spin–locking rf fields (B_{1H} and B_{1C}), the magnitude of which will satisfy the Hartmann–Hahn matching condition,

$$\gamma_H B_{1H} = \gamma_C B_{1C} \text{ [23]}$$

During this contact time, polarization transfer occurs allowing the rare ^{13}C spins to take on the magnetization and relaxation behavior of the abundant ^1H spins, leading to a sensitivity enhancement (up to four-fold based on the ratio of the ^1H and ^{13}C magnetogyric ratios) and a reduction in the pulse repetition time. Reducing the pulse repetition time allows a greater number of aquisitions to be accumulated per unit time, which yields a better S/N ratio. In instances where it may be difficult or impossible to record CP spectra because of weak ^{13}C–^1H coupling or short spin-lattice relaxation times in the rotating frame ($T_{1\rho H}$), direct polarization (Bloch decay) may be the only approach to recording solid-state NMR spectra.

MAGIC ANGLE SPINNING (MAS)

Line broadening in solid-state NMR is eliminated or averaged by both MAS and high-power ^1H decoupling. MAS involves mechanically rotating the sample at an angle of 54.7° (the magic angle) relative to the static magnetic field in order to simulate rapid molecular tumbling in solution. Rotating a solid sample at the magic angle to minimize line broadening requires that the sample be spun faster (in Hz) than the width of the CSA. High spinning rates are possible with current MAS probe technology, but complications can arise with CP and may require techniques such as ramped-amplitude CP (RAMP-CP) and variable-amplitude CP (VACP) to improve the efficiency of magnetization transfer from ^1H. Additionally, MAS can raise the sample temperature significantly if it is not controlled, and pressures at the periphery of the rotor may be thousands of times the ambient pressure. These stresses may induce phase transformations, loss of solvent, and other effects.

SIDEBAND SUPPRESSION

As shown in *Figure 9*, slower spinning rates can be used to avoid compromising the solid sample, but when CSA is incompletely averaged, spinning sidebands will appear in solid-state NMR spectra. These artifacts are separated from the centerbands by integer multiples of the spinning rate (in Hz) and can be readily identified as the peaks that shift in spectra acquired at different spinning speeds. Spinning sideband manifolds contain useful information but can interfere with the signals of interest and may be particularly problematic when one uses higher field instruments. The total suppression of spinning sidebands (TOSS) procedure is commonly used to eliminate spinning sidebands from solid-state NMR spectra.

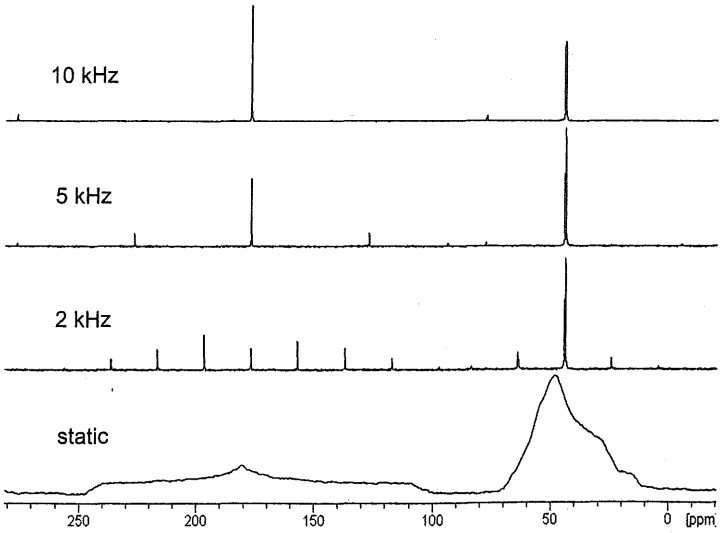

Figure 9. ^{13}C CPMAS NMR spectra of polycrystalline α-glycine collected at different sample spinning (MAS) rates. Spinning sidebands appear in solid-state NMR spectra when the sample spinning frequency is less than the width of the CSA pattern.

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HIGH-POWER ^{1}H DECOUPLING

High-power ^{1}H decoupling is used to further reduce the line broadening from dipolar coupling to ^{1}H spins in the solid state. Specialized hardware is required to deliver the rf power needed for ^{1}H decoupling in solids, an rf power that is two orders of magnitude greater than that required to remove scalar coupling in liquids. CW decoupling is commonly used in solid-state NMR, although two-pulse phase-modulated and small phase incremental alteration (SPINAL-64) decoupling are increasingly used to improve the sensitivity and resolution of dilute spin spectra.

Typically only ^{1}H–^{13}C heteronuclear dipolar interactions are significant when one acquires solid-state ^{13}C NMR spectra of organic materials. Homonuclear ^{13}C–^{13}C dipole–dipole and scalar coupling interactions are negligible. Because of their low natural abundance, the probability that two ^{13}C nuclei are in close proximity is very small. However, homonuclear ^{13}C–^{13}C dipolar coupling can be a concern for ^{13}C-labeled substrates.

EXPERIMENTAL SETUP

The basic setup for a CPMAS experiment will necessarily include magic angle setting, shimming, pulse calibration, Hartmann–Hahn matching, and spectral referencing, each of which is typically conducted on standard samples. Accurate measurement of the pulse lengths and associated rf power levels is essential for solid-state NMR experiments. Setting up the magic angle, shimming a CPMAS probe, and measuring its sensitivity for different tuning ranges are parts of probe setup and performance assessment. Shimming a solid-state NMR probe for MAS is more complicated than that for a solution probe because shim gradients are designed for the vertical orientation of solution NMR tubes. Solid-state NMR probes have no ^{2}H lock channel, so shimming must be performed manually. Because of the intrinsically broad peaks, shimming is not nearly as critical in solid-state NMR as it is in solution NMR.

Standard compounds used for setting the magic angle and optimizing pulse lengths and the associated rf power levels for CP are listed in *Table 6*. KBr is commonly used for magic angle adjustments, observing the ^{79}Br resonance and adjusting the sample spinning angle to maximize (to 10 ms and beyond) the duration of the rotational echo train in the FID. Liquid samples can be used to shim solid-state NMR probes, although solid adamantane is commonly used for this purpose. Adamantane, glycine, and hexamethylbenzene (HMB) are commonly used for Hartmann–Hahn matching and testing sensitivity. Typically, the reference sample for testing sensitivity is permanently packed into a rotor to ensure that the same amount of sample is used.

Table 6. Standard Samples Commonly Used for Setting Up CPMAS Experiments

Setup Procedure	Nucleus	Standard Sample(s)
Setting the magic angle	^{79}Br	KBr
Shimming	^{13}C	adamantane
Pulse calibration	^{1}H, ^{13}C	adamantane, HMB
Hartmann-Hahn matching	$^{1}H/^{13}C$	adamantane, HMB
Sensitivity	^{13}C	HMB, adamantane, α-glycine

Solid-state NMR spectrometers are generally used without field/frequency locking, so the resulting chemical shifts are less accurate than those for solutions. Calibration of the chemical shift can be done using either a primary or secondary standard. Spectral referencing is typically performed by sample replacement (external referencing). Standard compounds commonly used for spectral referencing in solid-state NMR are listed in *Table 7*. Note that glycine is polymorphic, so its crystal form should be ensured before its use in referencing spectra.

Table 7. Reference Compounds Commonly Used for Solid-State NMR

Nucleus	Primary Standard	Secondary Standard(s)	Chemical Shift in ppm from Primary Standard
^{13}C	TMS	HMB adamantane α-glycine	17.35 (CH_3) 38.48 (CH_2) 176.45 (carboxyl)
^{15}N	nitromethane	$NH_4{}^{15}NO_3$ $^{15}NH_4Cl$ α-glycine-^{15}N	–5.1 (NO_3) –338.1 –349.5
^{31}P	85% H_3PO_4	$CaHPO_4 \cdot 2H_2O$ (brushite)	1.4
^{29}Si	TMS	tetratrimethylsilylmethane	–1.4
^{19}F	CF_3Cl	perfluorobenzene	–166.4

General Test Procedure

Spectrometer performance should be demonstrated first for a reference sample as described in *Cross-Polarization Magic Angle Spinning (CPMAS) NMR Technique, Experimental Setup*. The magic angle, pulse lengths, and associated rf power levels for CP that are established using the reference compounds are sample independent. To obtain a CP spectrum of the sample, only the recycle delay needs to be chosen, followed by the contact time. When quantitative signal intensities are not required, an

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optimum recycle delay, i.e., one that affords the best S/N ratio, is $1.2T_{1H}$, and the contact time is generally that which provides the optimum S/N ratio or that which best shows the features of most interest. For quantitative CP, a recycle delay of at least five times the longest T_{1H} of a heterogeneous mixture is suggested to ensure full relaxation, and a full analysis of the CP signal as a function of contact time must be conducted. See the subsection *Quantitative Analysis.*

SAMPLE PREPARATION

Sample-handling procedures used in solid-state NMR are substantially different from those used in liquid NMR. Solid samples are packed in ceramic rotors that are capped with fluted drive tips specifically designed for MAS. Fine powders are typically tamped into MAS rotors, although solid plugs, e.g., whole tablets, can be cut to fit the exact inner dimensions of the rotor and can be inserted directly into the rotor. Crushing or grinding may be used to reduce the sample to a fine powder, but caution should be used in order not to induce phase transformations. Depending on the compressibility of the powder and the rotor volume, 40–400 mg of material is typically required to fill a sample rotor.

Physical Characterization

Specific components in heterogeneous systems can be searched based on unique nuclei or different NMR relaxation properties. The identification of crystalline and amorphous materials can be accomplished by comparison of the solid-state NMR spectrum of the sample preparation with that of a reference standard. Chemical shifts and relative peak intensities can be used in the comparison. Amorphous materials generally give good MAS spectra with broader peaks than those seen for crystalline materials. Highly crystalline samples typically give ^{13}C linewidths of the order of a few tens of Hz. The shape of the signals generally is between Lorentzian and Gaussian, a fact that should be recognized in deconvoluting overlapped spectra.

Relaxation

Relaxation parameters of interest in solids include spin-lattice relaxation (T_1), spin-lattice relaxation in the rotating frame ($T_{1\rho}$), spin-spin relaxation (T_2), and cross-relaxation (T_{CH}). In organic solids, ^1H spin diffusion is generally efficient so that pure compounds normally give single values for each of the relaxation times, T_1 and $T_{1\rho}$. For CP experiments, T_{1H} is used to establish the recycle delay between acquisitions. T_{1H} relaxation times can be measured using either progressive saturation or inversion recovery pulse sequences. In CP experiments, T_{CP} and $T_{1\rho H}$ characterize the magnetization build-up and decay, respectively. $T_{1\rho H}$ is measured via the ^{13}C signal using a delayed contact CP pulse sequence that has a variable delay time before the CP contact.

Quantitative Analysis

To quantitatively assess solid-state NMR spectra under CP conditions, extra measures must be taken. In addition to ensuring that the sample spinning axis is precisely set to the magic angle (54.7°) to minimize CSA broadening, the temperature and spin rate must be carefully controlled and the MAS probe properly tuned. Suggested recycle delays of $5T_{1H}$ are allotted between successive pulses to ensure that the magnetization has returned to its full equilibrium value. Both T_{CP} and $T_{1\rho H}$ relaxation must then be accounted for in the selection of the contact time. Typically, the CP contact time chosen is that which provides maximum sensitivity for the signals of interest. Quantitative analysis can be performed using either internal or external referencing methods. The use of internal standards can compensate for variability in sample volume and B_1 inhomogeneity throughout the sample.

By properly setting data acquisition parameters (recycle time, pulse widths, contact time, Hartmann–Hahn match, and decoupling power for each chemical system), signals can be obtained that are proportional to the number of nuclei producing them. For quantitative analysis, integrated signal intensities should be used rather than peak heights because linewidths in solid-state spectra often vary. When spinning sidebands are not eliminated by MAS, the intensity of the spinning sideband manifold must be added to the centerband intensity.

Spectral Editing

A key step in the analysis of any NMR spectrum is the assignment of individual resonances to unique phases and, in some cases, to specific atoms in the molecule. Special pulse sequences are available and may assist in simplifying CPMAS spectra and assigning signals. Dipolar dephasing, also known as nonquaternary suppression or interrupted decoupling, yields spectra that typically contain signals only from quaternary and methyl carbons. Spectral subtraction of dipolar dephasing spectra from normal CP spectra or short contact time CP may be used to produce subspectra that contain signals from methylene and methine carbons only. Polarization-inversion techniques can be used to identify methylene and methine carbons.

LOW-FIELD NMR

Low field NMR (LF-NMR), sometimes referred to as time domain NMR (TD-NMR), experiments are performed by measuring relaxation, relaxometry, or diffusion. Instrumentation for these applications is based on low-field permanent magnet technologies that operate in the 2–25 MHz frequency range. Inexpensive stationary bench-top and portable TD-NMR spectrometers are commercially available. Typical bore sizes are 10–50 mm in diameter. A recent development in spectrometer design uses a mobile mouse probe as an alternative to a stationary magnet. This design allows analysis of samples of unrestricted size.

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Most TD-NMR applications are based on simple pulsing sequences, including FID, Hahn–echo, Carr–Purcell– Meiboom–Gill, and solid–echo acquisition. The choice of pulse sequence depends on the physical and chemical properties of the sample as well as the information that is desired from the experiment. These systems can be used to measure longitudinal (spin-lattice, T_1) and transverse (spin-spin, T_2) relaxation times. Diffusion properties of compounds can be exploited using pulsed field gradient (PFG-NMR) experiments.

The classical application of relaxometry is for the determination of food product components based on differences in longitudinal and transverse relaxation times of water, fats, and proteins.

Relaxivity

The magnitude of a substance's capacity to enhance the relaxation rate of a nucleus is referred to as relaxivity, expressed in units of $sec^{-1}mM^{-1}$. An example of such a substance commonly used in NMR spectroscopy is paramagnetic chromium acetylacetonate. Paramagnetic species are used in the medical industry as contrasting agents for magnetic resonance imaging. The relaxivity of a substance is determined experimentally by measuring the spin-lattice relaxation time (T_1) of a test substance and plotting $1/T_1$ against the concentration in units of mM (mmol/L). The slope of the curve is the numerical relaxivity.

⟨1771⟩ OPHTHALMIC PRODUCTS—PERFORMANCE TESTS

INTRODUCTION

This chapter provides information on performance tests to assess drug release from finished ophthalmic products. These tests are applicable to products that have an extended-release mechanism (beyond 1 day); the dissolution/drug release rate is rate limiting for absorption and is expected to provide a controlled therapeutic response. Examples of such products include intraocular matrix-type and polymer-based bioerodible implants, non-bioerodible intraocular matrix and reservoir implants, intraocular injectable suspensions/colloidal systems, intraocular in situ–forming depots and gels, punctal plug-based delivery systems, non-biodegradable drug release devices (e.g., drug-coated stents, drug-coated contact lenses), biodegradable and non-degradable ocular inserts/bioadhesives (for cul-de-sac or conjunctival-sac applications), and other such dosage forms. For products having a localized and immediate response when applied to the eye (e.g., topically applied dosage forms, including dispersed systems, having very short residence time for absorption), a dissolution/drug release test may have no practical value.

Application of a dissolution/drug release test to assess performance as a surrogate for in vivo testing should be considered only with appropriately validated in vivo–in vitro correlations (see *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩). Dissolution/drug release tests are used as a quality control tool for specific product attributes (e.g., burst release from matrix-type, biodegradable, polymeric systems). These tests may have the ability to distinguish between different lots of a drug product having one or more formulation and/or process changes.

Changes may be related to the drug substance(s) or excipients present in the formulation, physical and/or chemical attributes of the finished formulation, critical manufacturing variables, shipping and storage effects, aging effects, or other formulation and/or process factors critical to the performance of the ophthalmic product. Such changes may affect the performance characteristics of the dosage form when applied to the eye. Consideration should be given to whether processing or components are intended to provide for prolonged exposure to any part of the eye and whether a dissolution/drug release test could be used to demonstrate this effect.

PERFORMANCE TESTS (DISSOLUTION/DRUG RELEASE)

Depending on the design and release mechanism of the dosage form, the dissolution/drug release test can be developed using any apparatus described in *Dissolution* ⟨711⟩ or *Drug Release* ⟨724⟩. Novel dosage forms may require the use of noncompendial equipment and/or conditions. Any dissolution/drug release test should be discriminative for the intended critical quality attributes of the product and should be properly validated (see *The Dissolution Procedure: Development and Validation* ⟨1092⟩). The test conditions should reasonably mimic the method of administration of the product and in vivo conditions to establish, if possible, an in vivo–in vitro correlation that can be used to predict in vivo performance of the product.

⟨1782⟩ VIBRATIONAL CIRCULAR DICHROISM SPECTROSCOPY—THEORY AND PRACTICE

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1. INTRODUCTION

Chirality is a ubiquitous aspect of the world of three spatial dimensions. Molecules that possess sufficient structural complexity so that their mirror-image structures are non-superimposable are termed chiral. For chiral pharmaceutical molecules, two important structural measures critical to their physical characterization are absolute configuration (AC) and enantiomeric excess (EE), also termed enantiomeric purity. The AC of a chiral molecule specifies its three-dimensional structure in space and distinguishes it from its mirror-image structure. Structures related by mirror symmetry are known as "enantiomers". The AC of a particular enantiomer is critical to its action as a pharmaceutical agent because mirror-image structures, i.e., enantiomers, have different therapeutic effects, both desired and undesired. The EE of a sample specifies, usually as an excess percentage, the relative amounts of enantiomers. An EE of 100% is a pure enantiomer (100% enantiomeric purity), 0% is a racemic mixture with equal amounts of enantiomers and no excess, and −100% specifies a pure sample of the opposite enantiomer with respect to the EE definition.

The principal chapter that addresses molecular chirality in the *USP–NF* is *Optical Rotation* ⟨781⟩. The optical rotation (OR) of a sample is the measure of the angle of rotation of a plane of polarized light by a chiral sample at a particular wavelength, typically in the visible or near-UV region. The AC and EE of any sample are determined by a measure of its OR if the measurement has been previously calibrated using a sample of known AC and EE. The sign of the OR determines the larger enantiomer amount, and the magnitude of the OR determines the EE, where 100% EE corresponds to the maximum OR possible for the chiral molecule under consideration. Although OR is a simple, well-established method for determining AC and EE relative to their known calibrations, not all molecules have measurable OR, particularly with limited sample quantities in which the presence of chiral impurities cannot be determined by an OR measurement. The OR is simply a number with no structural information about the sample molecule.

In the past several decades, the AC of an unknown chiral molecule has been determined using the Bijvoet method of single-crystal X-ray crystallography. The Bijvoet method requires a single high-purity crystal of the molecule with a single chiral phase. Analysis by this method is not always possible, for example, for noncrystallizable liquids or insoluble solids, and in many cases analysis using the Bijvoet method requires lengthy efforts and time to achieve crystallization.

This chapter presents a technique for the determination of AC that relies on vibrational circular dichroism (VCD), a procedure that is now widely used throughout the pharmaceutical industry for chiral molecules for which the AC is unknown. The technique involves comparing the measured VCD spectrum for a chiral molecule to the quantum chemistry calculation for the same molecule. If the measured and calculated VCD spectra show agreement on the principal features and their signs, this means that the AC of the physical sample is the same as the AC of the structure used in the calculation. If the signs are opposite, the AC of the sample is the opposite of that used in the calculation.

As described below, statistical methods have been developed for assessing the degree of confidence that the AC has been correctly determined by the VCD method. Usually the AC of a chiral molecule is specified by connecting its structural chirality, labeled with *R* or *S* for chiral centers or *P* or *M* for chiral axes, to the sign of its measured OR as (+) or (−). Once the AC of a molecule has been determined by VCD, for example, (S)-(−)-α-pinene, its VCD spectrum can become a reference standard of the AC of this molecule, and the AC of any subsequent sample of this molecule can be determined by comparison to its VCD reference standard. An important advantage of determining AC by VCD is the spectral richness of a VCD spectrum that supports the simultaneous determination of the structural identity of the molecule and its absolute chirality. In contrast to OR, every chiral molecule has a VCD spectrum, because all molecules have infrared (IR) absorption bands, each one of which acts as a chromophore for a VCD spectrum. VCD also is sensitive to molecular conformations. As demonstrated below, information about the conformation of a chiral molecule is obtained as a bonus from the VCD determination of AC, but AC is the principal informational content of chiral pharmaceutical molecules. An extensive body of literature describing the AC of biological molecules has been created during the past 30 years and can be used as a basis for determining secondary and higher-order structural states of biological drug substances. Stereospecific methods using VCD can be developed to characterize the production, formulation, and stability of biopharmaceutical products.

This chapter also presents a method for the determination of EE using VCD. EE determination with VCD takes advantage of the fact that the magnitude of a VCD spectrum, measured as the circular polarization absorbance difference for a constant parent IR absorbance spectrum, is directly proportional to EE with no offset. Thus, the maximum relative size of a VCD spectrum is obtained for an EE of 100%, is zero for an EE of 0% (racemic mixture), and is maximum with opposite signs (all positive VCD bands are negative and all negative VCD bands are positive) for an EE of −100%. The advantage of VCD EE determination is that the EE of any molecular sample can be determined by a single VCD measurement once the relative size of VCD for 100% EE (or any %EE value) is known for that molecule. Separation of enantiomers with chiral chromatography is not needed.

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2. DEFINITION OF VIBRATIONAL CIRCULAR DICHROISM

VCD is defined as:

$$\Delta A = A_L - A_R$$

ΔA = the difference in the IR absorbance
A_L = sample absorbance A for left circularly polarized (LCP) radiation
A_R = sample absorbance A for right circularly polarized (RCP) radiation

Unpolarized IR absorbance intensity is defined as the average of LCP and RCP intensities:

$$A = (A_L + A_R)/2$$

The IR intensities of enantiomers are identical, whereas enantiomers have equal and opposite-signed VCD intensities.

3. VCD INSTRUMENTATION

The measurement of a VCD spectrum and its parent mid-IR spectrum is based on Fourier transform-IR (FT-IR) absorption spectroscopy. The optical–electronic layout for the measurement of VCD using an FT-VCD spectrometer is illustrated in *Figure 1*. The output beam of an FT-IR spectrometer is optically filtered and then linearly polarized before passage through a photoelastic modulator (PEM). The zinc selenide (ZnSe) PEM, typically used for VCD measurement, changes the polarization state of the beam between LCP and RCP at a frequency of 37 kHz. A sample solution in a standard IR cell is placed in the beam, and a liquid nitrogen-cooled detector of mercury–cadmium telluride (MCT) detects the transmitted beam. The detector produces signals in two frequency ranges: 1) I_{DC}, in the range of 1–2 kHz, represents the FT-IR interferogram; and 2) I_{AC}, centered at 37 kHz, represents the FT-VCD interferogram. The FT-IR interferogram may be directly Fourier-transformed to yield the IR spectrum. After synchronous demodulation (using a lock-in amplifier or purely numerical processing) with a reference at 37 kHz, the VCD interferogram can be Fourier-transformed to yield the VCD spectrum. Both the IR and VCD spectra are presented in dimensionless absorbance units, A, and hence the ratio of the VCD to the IR intensity for any band in the spectrum yields the dimensionless anisotropy ratio for that vibrational transition. *Figure 1* illustrates the minimum setup for VCD measurement, but technically advanced instruments use two PEMs to increase baseline stability and two sources to increase signal quality or reduce sample measurement time. Measurement of VCD in other spectral regions, for example the hydrogen-stretching or near-IR, can be carried out with appropriate changes in sources, polarizers, filters, PEM, cells, and detectors.

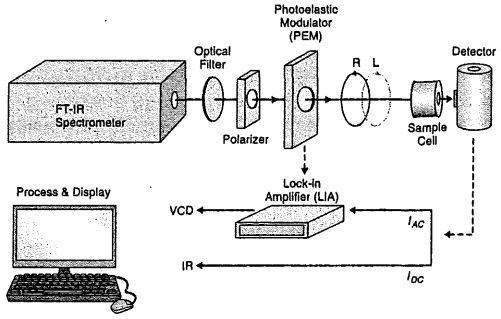

Figure 1. Diagram illustrating the typical features of an FT-VCD spectrometer for the measurement of the IR and VCD spectra of a chiral sample.

4. MEASUREMENT OF VCD SPECTRA

Figure 2 shows the IR and VCD spectra for (+)-*R*- and (–)-*S*-α-pinene as a neat liquid measured at 4 cm^{-1} spectral resolution for 1 h. Also shown are the stereostructures of the two opposite enantiomers (+)-*R*- and (–)-*S*-α-pinene. This figure shows that the IR spectra are nearly identical (superimposed dash and solid traces) for these enantiomers and that IR is therefore blind to chirality. By contrast, the corresponding VCD spectra have intensities that are equal in magnitude with opposite signs for each band in the spectrum. The two stereostructures for α-pinene are presented in a form that makes clear their mirror symmetry, and it follows that mirror-symmetric pairs of chiral molecules have mirror-symmetric VCD spectra about the zero baseline. The VCD baseline for these spectra is offset slightly above zero and can be baseline corrected as discussed below. Inspection of the intensity scales for the IR and VCD spectra reveals that the VCD intensities are approximately four orders of magnitude smaller than the corresponding IR intensities. Finally, each band in the IR spectrum has a corresponding VCD band. The richness of the resolved spectral bands in the VCD spectrum, each one representing what has traditionally been called a chromophore in electronic circular dichroism (ECD), gives VCD its exceptional combination of structural richness and stereochemical specificity.

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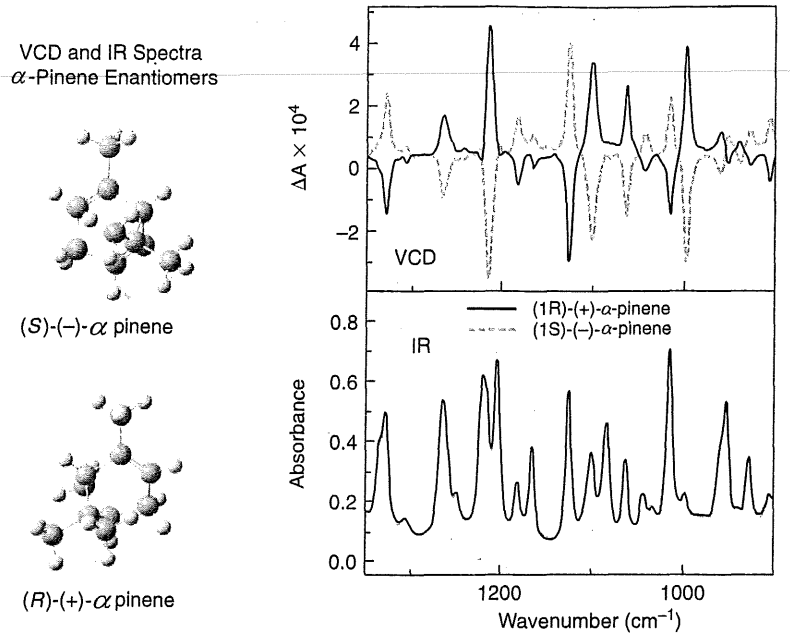

Figure 2. IR (lower) and VCD (upper) of (1*S*)-(–)-α-pinene (dash) and (1*R*)-(+)-α-pinene (solid). To the left are presented the quantum chemistry-optimized stereostructures of (1*S*)-(–)-α-pinene (upper) and (1*R*)-(+)-α-pinene (lower). The IR and VCD spectra were measured at 4 cm^{-1} spectral resolution, and the VCD spectra were averaged for 1 h.

Sampling requirements for VCD are similar to those needed to obtain a good IR spectrum. Ideally, as shown in *Figure 2*, analysts choose a combination of path length and sample concentration to obtain an average IR absorbance value *A* in the range between 0.2 and 0.8 and concentrations between 0.1 and 1.0 M, depending on the molecular weight of the sample. Typical path lengths for organic solvents are in the range of 50–100 µm, and sample quantities needed are typically 5–10 mg, although amounts as small as 2 mg are possible. Typical solvents for VCD measurement of organic molecules are deuterated solvents that have reduced solvent absorption in the mid-IR region. Besides hydrogen-free solvents, such as carbon tetrachloride (CCl_4), other commonly used solvents for VCD are deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-d_6).

IR and VCD spectra can be plotted either in absorbance units, as *A* and ΔA, that reflect the sampling conditions, or in molar absorption coefficients, ε and $\Delta\varepsilon$, that remove the concentration and path-length dependence of the intensity to give a molecular-level property that can be compared quantitatively to calculated IR and VCD intensities. The conversion between these two sets of quantities is given by:

$$A = \varepsilon C \times l$$

A	= IR absorbance
ε	= molar absorption coefficient
C	= concentration (mol/L)
l	= path length (cm)

and

$$\Delta A = \Delta\varepsilon C \times l$$

ΔA	= difference in the IR absorbance
$\Delta\varepsilon$	= difference in molar absorption coefficient

A useful dimensionless measure of the intrinsic strength of a VCD band is the anisotropy ratio, defined as $g = \Delta A/A = \Delta\varepsilon/\varepsilon$. Including the spectral frequency dependence of IR and VCD, the measured spectra are expressed respectively either as $A(\nu)$ and $\Delta A(\nu)$ or as $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$.

VCD spectra require baseline corrections for instrumentation and solvent intensity. Deviations from a perfectly flat baseline with zero offset must be removed before a final calibrated VCD spectrum is complete. Solvent baseline correction can be achieved for VCD spectra by subtraction of the VCD spectrum of the solvent in the same sample cell. If the opposite enantiomer or racemic mixture of the sample is available, baseline correction can be achieved as one-half the difference between these two equal and opposite VCD spectra in the case of enantiomers or as simple subtraction in the case of the racemic mixture. Baseline deviations are caused by the unavoidable presence of linear birefringence in the optical elements of the instrument and the sample cell that can become important for all but the largest VCD intensities. An example of a baseline-corrected VCD spectrum is presented in *Figure 3*, along with the corresponding IR and VCD noise spectrum. The spectrum of this sample of neat

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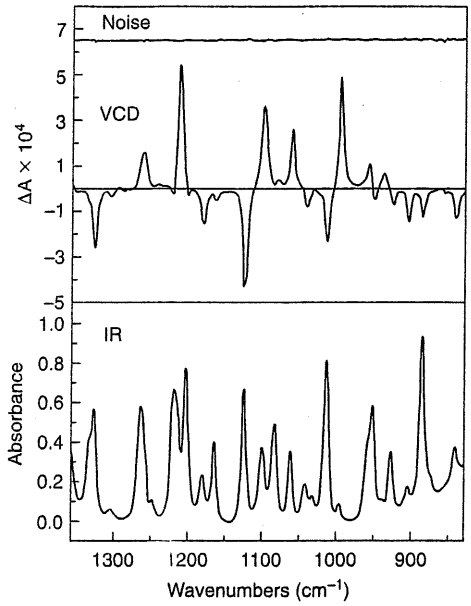
(–)-(*S*)-α-pinene was collected for a period of 1 h at 4 cm^{-1} spectral resolution in barium fluoride (BaF_2) with a path length of 75 µm. The noise spectrum is less than 1×10^{-5} absorbance (*A*) units across the spectrum. The IR, VCD, and noise spectra presented in *Figure 3* may be taken as a validation standard for the performance of a mid-IR FT-VCD instrument, as described in *Vibrational Circular Dichroism Spectroscopy* ⟨782⟩.



Simultaneous, two enantiomers, total 3.5 hour

Figure 3. IR absorbance A (bottom), baseline-corrected VCD (middle), and VCD noise spectrum (top) for (–)-(1*S*)-α-pinene as a neat liquid averaged for 1 h at 4 cm^{-1} spectral resolution and in a 75-µm path-length barium fluoride cell.

5. QUALITATIVE AND QUANTITATIVE ANALYSIS

5.1 Ensure That Signs and Intensities Are Correct

Qualitative analysis can be carried out with VCD by comparing the VCD spectrum of an unknown to a reference spectrum of that molecule to identify common features and to confirm that the VCD spectrum of the sample is a close match to the reference spectrum.

Quantitative analysis using VCD involves measuring the VCD intensity of the sample molecule and comparing it to a standard reference spectrum for that molecule. In this way, the concentration and the %EE of the sample can be determined as follows. The intensity of the IR spectrum relative to the IR standard of the sample determines the concentration for measurements with the same path length. After normalizing the IR intensity of the unknown sample to the standard, the relative intensities of the VCD determine the %EE of the sample. Such comparisons need to be carried out with all sampling conditions being the same, including the solvent and instrument spectral resolution.

To determine that the VCD intensities are correct, the uncalibrated VCD intensities must be calibrated. This is accomplished by placing a multiple-wave plate in the sample position, followed by a polarizer that is either parallel or perpendicular to the instrument polarizer in *Figure 1*. Measurement of the VCD spectrum of this multiple-wave plate set-up for two settings of the wave plate and polarizer that differ by 90° produces two sets of curves that have crossing points. The curve connecting the crossing points follows the shape of a first-order Bessel function and represents unit VCD intensity. Dividing this curve into the uncalibrated VCD spectrum produces a VCD spectrum with the correct calibrated intensities. The two calibration curves and their crossing-point curve are illustrated in *Figure 4*.

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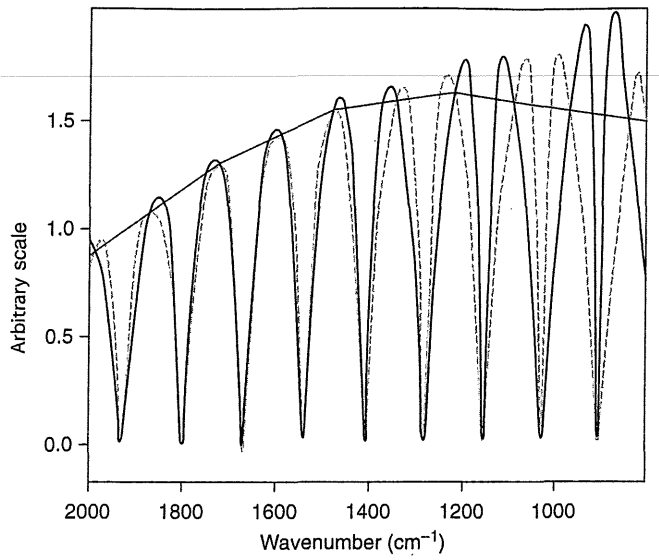

Figure 4. Calibration curves for VCD intensity generated by placing a multiple-wave plate and a polarizer in the IR beam and connecting the crossing points with a curve representing unit VCD intensity for maximum VCD intensity at 1200 cm^{-1}.

5.2 Determination of the Noise Level at Each Point in the Spectrum

A critical factor in quantitative analysis using VCD is the degree of accuracy of the spectrum as given by the VCD noise level and baseline stability of the measurement. The noise level depends on a number of factors such as the D* value of the detector, the amount of radiation passing through the sample from the source, and the amount of absorption of the sample. The noise level is obtained by dividing the VCD measurement into two halves. Addition of these halves gives the VCD spectrum, including noise, whereas subtraction eliminates the VCD features and leaves the equivalent noise spectrum. An example of a VCD noise spectrum is given in *Figure 3*, which shows, from bottom to top, the IR, VCD, and VCD noise spectra for a measurement of (–)-*S*-α-pinene. The VCD noise level can be reduced by increasing the measurement time through co-addition of the spectra, as desired.

5.3 VCD Baseline Accuracy

A second critical factor in the measurement of a VCD spectrum is the characteristics of the VCD baseline, namely its location, straightness, and stability. The VCD zero intensity line between positive and negative VCD intensities needs to lie as close as possible to the electronic zero of the measurement (zero of spectral display). Due to the sensitivity of the VCD intensity scale, some offset of the baseline from electronic zero almost always occurs, as discussed above in reference to the need for baseline correction of the VCD spectra of α-pinene in *Figure 2* and as carried out for *Figure 3*. Use of α-pinene as a validation standard is advantageous, because α-pinene has an unusually large intensity and thus, its spectrum is relatively easy to measure on a regular basis. α-Pinene can also be used as a measure of the baseline of a VCD measurement relative to the electronic zero of the measurement. This is illustrated in *Figure 5*, where the VCD spectra of (–)-α-pinene (*dash*) and (+)-α-pinene (*black*) are presented along with the VCD spectrum of a racemic mixture [equal quantities of (–)-α-pinene and (+)-α-pinene, hence cancelling the VCD intensity] of α-pinene (*gray*). The VCD spectrum of the racemic mixture of α-pinene is the VCD baseline for this measurement. A typical standard for baseline quality is deviations from electronic zero of <10% of the maximum separation of positive and negative VCD intensity of the neat α-pinene spectrum, or approximately 1×10^{-4}. The baseline illustrated in *Figure 5* satisfies this standard to within 5%. The baseline at 1350 cm^{-1} is slightly above electronic zero and has maximum excursions away from electronic zero of <0.00005, or 5×10^{-5} at 1170 cm^{-1}, which can be compared to the maximum positive and negative intensity values of (–)-α-pinene of $+5 \times 10^{-4}$ at 1220 cm^{-1} and a negative peak value of -4×10^{-4} at 1130 cm^{-1}.

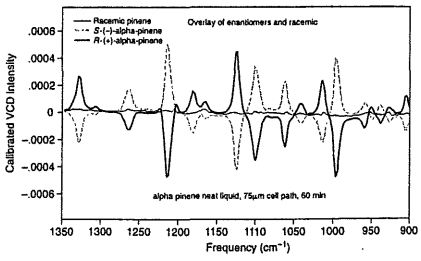

Figure 5. VCD spectrum of *R*-(+)-α-pinene (black), *S*-(–)-α-pinene (dash), and racemic (gray) α-pinene as a neat liquid for a 60-min measurement in a 75-µm path-length barium fluoride cell.

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5.4 VCD Baseline Absorption Artifacts and Single-Enantiomer Measurement Capability

For tests of baseline accuracy and stability, the sample camphor in carbon tetrachloride solution is used as a qualification standard and provides a more stringent test than α-pinene, because the VCD spectrum of camphor is approximately one-fifth the magnitude, relative to the IR absorbance spectrum, of that of neat α-pinene. The VCD spectra of both enantiomers, as well as two measures of the VCD baseline, are provided in *Figure 6*. It is clear, as in *Figure 2* and *Figure 5*, that the VCD spectra of enantiomers, in this case (+)-camphor and (−)-camphor, are equal in intensity and opposite in sign relative to the VCD baseline to within the noise level of the measurement. The two VCD baselines provided are both zero VCD measurements. The VCD spectrum of racemic camphor [equal mixture of (+)-camphor and (−)-camphor] is the true VCD baseline for the individual (+)-camphor and (−)-camphor spectra, even if there are offsets (artifacts) due to absorption bands in the IR spectrum of camphor. The absence of such artifacts to within the noise level of the measurement is demonstrated by the congruence of the VCD spectrum of the racemic mixture and that of the solvent, carbon tetrachloride. Because carbon tetrachloride has no significant level of absorbance in the region of the spectrum displayed, the agreement of these two baselines within the noise level demonstrates the absence of baseline artifacts in the VCD spectrum of racemic camphor, and therefore, for the VCD measurement of either of the two enantiomers of camphor.

Achieving a VCD baseline that is free of absorption artifacts is usually done by optical alignment of the VCD instrument such that the baseline is as close as possible to the true electronic zero of the instrument of the measurement. In *Figure 6*, the VCD baseline can be seen to wander above and below zero across the spectrum, but in this case, the deviations from the electronic zero are small (approximately 2×10^{-5}) and <20% of the positive and negative maximum VCD intensities of the spectrum (approximately 1×10^{-4}). This value of deviation is consistent with the maximum baseline magnitudes discussed above for α-pinene and displayed in *Figure 5*, because α-pinene has a much larger (approximately 5×) VCD spectrum, relative to its IR spectrum, than camphor. The baselines for camphor in *Figure 6* are noisier than that of α-pinene in *Figure 5*, because the VCD spectrum scale is smaller and the measurement time is 20 min instead of 1 h. As in *Figure 3*, the VCD baseline can be corrected by subtraction of an appropriate baseline spectrum, racemic or solvent. In *Figure 7*, the baseline-corrected VCD spectra for both enantiomers and the racemic mixture have been obtained by subtraction of the VCD spectrum of the solvent carbon tetrachloride. As a result the VCD zero baseline and the instrument electronic zero are the same to within the noise level, and the mirror symmetric properties of these spectra are even more apparent. This VCD test verifies that by subtracting the VCD spectrum of the solvent, an accurate VCD spectrum of camphor can be obtained using only one enantiomer, either the (+)- or (−)-enantiomer, and hence, the instrument possesses a single-enantiomer VCD measurement capability.

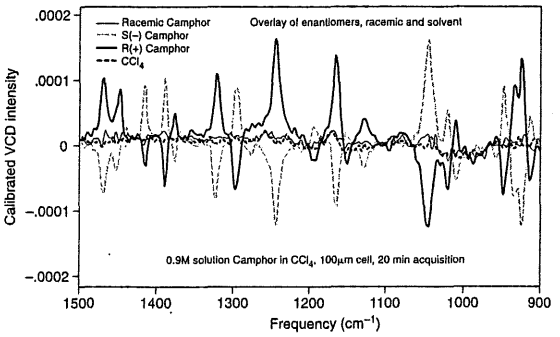

Figure 6. VCD spectrum of *R*-(+)-camphor (black), *S*-(−)-camphor (gray dash), and racemic camphor mixture (gray) as a 0.9 M solution in carbon tetrachloride. Also provided with the same cell and path length is the VCD spectrum of pure carbon tetrachloride (black dash). The sampling conditions were a spectral collection time of 20 min with a 100-μm path-length barium fluoride cell.

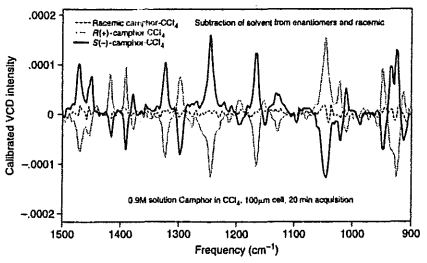

Figure 7. Solvent baseline-corrected VCD spectrum of *R*-(+)-camphor-carbon tetrachloride (gray), *S*-(−)-camphor-carbon tetrachloride (black), and racemic camphor-carbon tetrachloride mixture (dash) of the VCD spectra shown in *Figure 6*.

5.5 VCD Measurement Stability and Noise Level Reduction

Comparison of VCD measurements of camphor for 20 min to that measured over a period of 4 h in blocks of 20 min permits evaluation of instrument measurement stability as well as the reduction of noise level over time. In *Figure 8*, 12 such 20-min VCD spectra measured in 4 h are presented to show the stability of a VCD spectrum over time. It is clear that there are no

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deviations of VCD spectra outside the noise level of any one 20-min spectrum, and hence, there is no long-term drift in the VCD baseline over this time period.

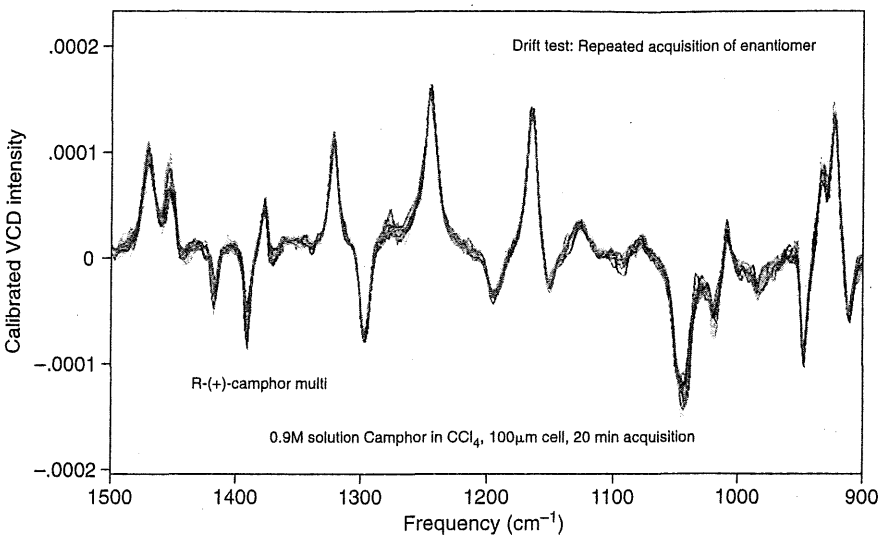

Figure 8. Superposition of 12 20-min VCD spectra of *R*-(+)-camphor under the conditions of *Figure 6* and *Figure 7*.

If the 12 blocks of 20-min VCD spectra in *Figure 8* are averaged, the resulting 4-h VCD spectrum can be compared to a 20-min VCD spectrum of the same sample to demonstrate the noise reduction that is achieved upon signal averaging, provided there are no other systematic noise sources in the VCD spectrometer. In this case, the noise reduction should be a factor of the square root of 12, or approximately 3.5. Thus, the signal-to-noise ratio should improve by a factor of 3.5. Such a comparison is provided in *Figure 9*.

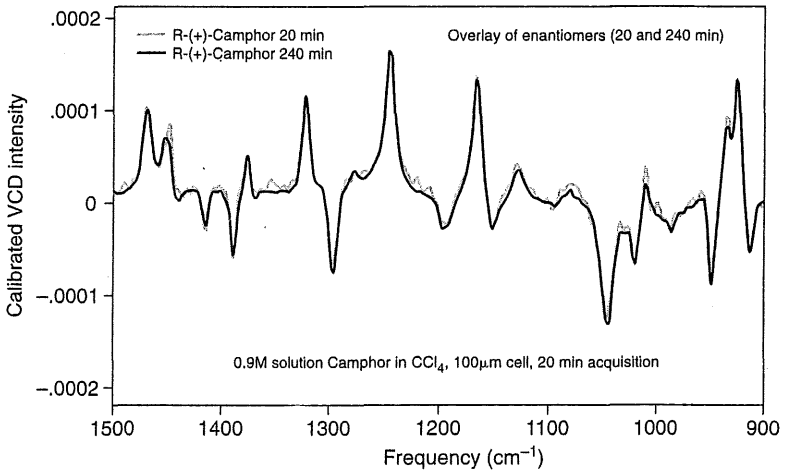

Figure 9. Comparison of the 4-h average of 12 20-min VCD spectra [*R*-(+)-camphor 240 min (gray)] to one 20-min measurement of camphor [*R*-(+)-camphor 20 min (black)], under the conditions of *Figures 6*, *7*, and *8*, showing improvement in signal-to-noise ratio with increased collection time.

The noise level can be visualized directly on a 5× more sensitive scale to better evaluate the noise levels by comparing the noise spectrums, as illustrated and described in *Figure 3*, for the 4-h and 20-min VCD measurements. This is presented in *Figure 10*.

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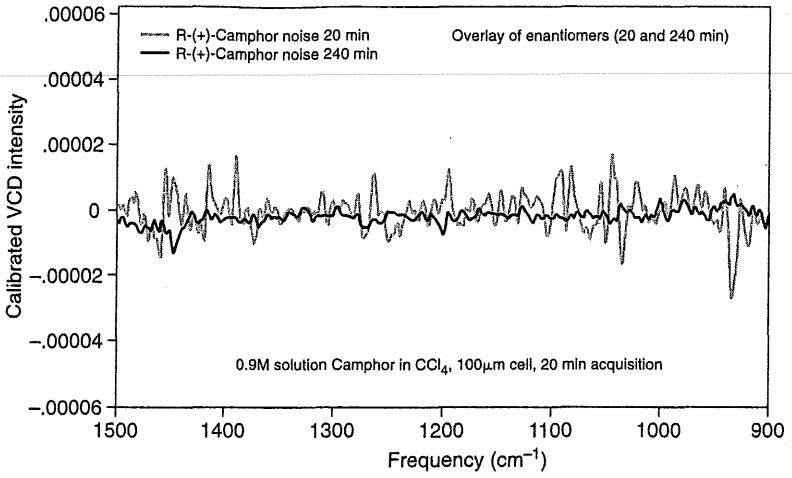

Figure 10. Comparison of the VCD noise spectra of *R*-(+)-camphor for one 20-min measurement [*R*-(+)-camphor noise (gray)] versus a 4-h measurement [*R*-(+)-camphor noise (black)] under the conditions of *Figures 6*, *7*, *8*, and *9*, where the intensity scale has been expanded to accommodate noise spectra that are 5× enlarged for better viewing from the noise levels in the previous four figures.

6. DETERMINATION OF ABSOLUTE CONFIGURATION

The IR and VCD spectra of a sample are measured in solution in a matter of minutes to hours without the requirement of prior crystallization or high sample optical purity. The AC of an unknown sample is determined by comparison of its measured VCD spectrum to a VCD quantum chemistry calculation of the same molecule, where the AC used in the calculated VCD spectrum is chosen arbitrarily. If the signs of the bands in the measured VCD spectrum match those in the calculated VCD spectrum, the AC of the sample is identical to that used in the calculation. If the signs are opposite, then the sample has the opposite AC compared with that calculated. An example of the determination of the AC of a pharmaceutical molecule is shown in *Figure 11*. The AC of mirtazapine, which is sold in racemic form, is determined by comparison of the measured IR and VCD spectra of the (–)-enantiomer to the calculated IR and VCD spectra of the *R*-enantiomer. The close match in VCD signs—vibrational frequencies, intensities of the observed and calculated spectra, and IR frequencies and intensities—demonstrates clearly that the AC of mirtazapine is (–)-*R*. Also shown in *Figure 11* is the stereospecific quantum chemistry calculated structure of *R*-mirtazapine.

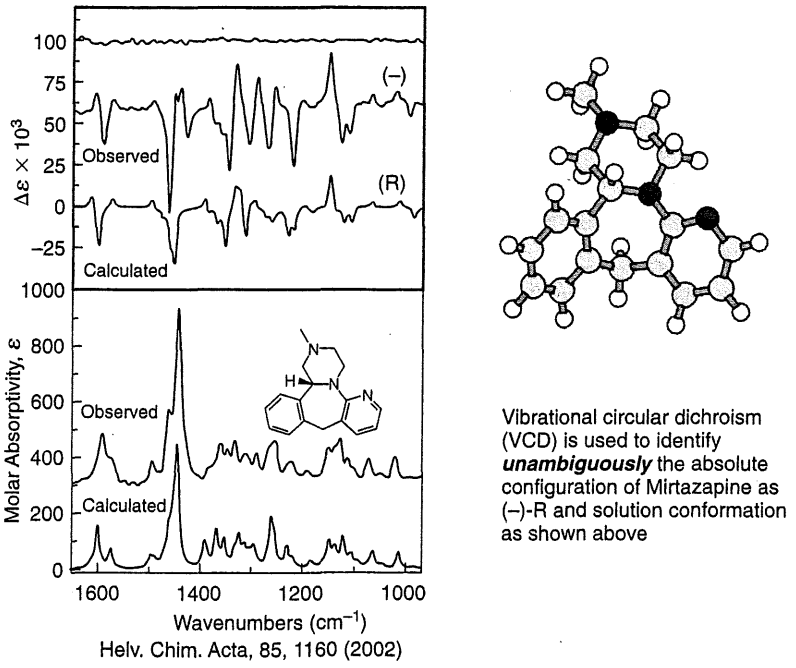

Figure 11. Comparison of the measured (observed) and calculated IR and VCD spectra of mirtazapine, allowing assignment of its AC to (–)-*R*. Reproduced in part with permission from John Wiley & Sons.

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If the AC of a molecule has previously been established, for example, by X-ray crystallography, a VCD calculation of the molecule is not necessary (but could be performed as a check of the X-ray assignment), and the measured VCD spectrum of the molecule may be taken as a reference standard for the AC of the molecule, supported, for example, by USP. For a molecule with unknown structural chirality, the determination of its AC by VCD requires a comparison of the measured and calculated IR and VCD spectra, $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$, as shown in *Figure 11*. The key point of the comparison is correlating the major VCD bands to determine whether they have the same or opposite signs. The IR spectrum is a guide in the process. Seeing a close correspondence between the calculated and measured IR spectra provides confidence that a good vibrational force field has been calculated and the most important vibrational bands are correlated. If a corresponding correlation of the signs of the major VCD bands can be made, as in *Figure 11*, then the AC has been determined.

When the measured and calculated VCD spectra are compared, the magnitudes of the corresponding intensities are not critical. What is important is the relative intensities of the bands in the two spectra, and in fact, that the comparison of measured and calculated spectra can be made using different sets of intensities for $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$ for the calculated spectra and $A(\nu)$ and $\Delta A(\nu)$ for the measured spectra. In general, an exact match of the measured and calculated band frequencies is not expected because of several limitations of the calculations. Examples of these limitations include the assumption of the harmonic approximation when some anharmonicity is present in the measured spectra, the need to ignore the effects of solvent interactions with the chiral solute molecule, limitations in the size of the basis set used for the quantum calculation, and the choice of density functional required for the density functional theory (DFT) calculation.

7. CALCULATION OF VCD SPECTRA

To determine the AC of a molecule with unknown chirality using the VCD method as an alternative to, or supplement to, X-ray crystallography, analysts conduct a quantum chemistry calculation of the IR and VCD intensities of the molecule. Since the late 1990s, commercial DFT software has been available and can be used effectively by a trained user because it does not require special expertise or extensive experience in quantum chemistry calculations. For example, VCD calculations are now part of some undergraduate chemistry curricula. The following steps are required to perform a DFT calculation of IR and VCD:

1. The stereospecific structure of the molecule is entered through a visual graphics interface.
2. The quantum chemistry program then automatically performs the following steps:
   - A. The geometry is optimized to a minimum energy conformation.
   - B. A force field is calculated that determines the vibrational modes of the molecule, with 3N-6 modes for a molecule with N atoms.
   - C. Atomic polar tensors that yield the IR spectrum are calculated.
   - D. Atomic axial tensors that yield the VCD spectrum are calculated.
3. The output of the calculation consists of a table where for each mode *i* there is a frequency, ν_i, IR intensity (dipole strength), D_i, and VCD intensity (rotational strength), R_i.
4. Commercially available programs calculate the IR and VCD spectra for the molecule using the following steps:
   - A. A line shape, $f(\nu)$, is applied to each dipole and rotational strength value, D_i and R_i, centered at each vibrational frequency, ν_i.
   - B. Summation of these normal mode line-shaped intensities automatically produces the final calculated IR and VCD spectra $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$ that can be compared directly to the corresponding measured IR and VCD spectra.

Another important factor that often is encountered in the assignment of the AC of pharmaceutical molecules and natural products (because of the structural complexity of these types of molecules) is the presence of more than one important conformer under the measurement conditions. The fractional populations of different conformers can be predicted by using their relative calculated energies in the Boltzmann distribution. The lowest-energy conformer has the highest fractional population and so on, to increasingly less-populated conformers with higher relative energies. To calculate the VCD, one must calculate the IR and VCD of each important conformer and then add these spectra together, weighted by the fractional Boltzmann population. Usually, one can ignore the spectral contributions of conformers that account for less than a few percent of the total population.

Programs for calculating VCD and IR spectra to the level of accuracy sufficient for comparison to measured IR and VCD are available commercially from a variety of sources. First, one needs a program for evaluating and finding the geometry of all the lowest-energy conformations of a chiral molecule. A variety of programs using molecular mechanics are available for this purpose. Second, one needs a full quantum chemistry software program for calculating the ab initio force fields, vibrational frequencies, and VCD and IR intensities for each of the lowest-energy conformers. A common chemical model of sufficient accuracy for AC determination is DFT, using a basis set minimum of 6-31G(d), and a choice of hybrid functionals, such as B3LYP or B3PW91. Higher-level basis sets and alternative choices of hybrid functionals may be made. Finally, one needs a software method for comparing measured and calculated VCD and IR spectra to assess the degree of spectral agreement between the measured and calculated spectra, and hence the level of confidence that the correct assignment of AC has been predicted. Additional details are described below.

In *Figure 12*, the AC of the bioactive enantiomer of the analgesic (*S*)-(+)-ibuprofen is determined by comparing the measured and calculated IR and VCD spectra. The calculated IR and VCD are the sums of the IR and VCD conformer spectra, one for each conformer, weighted by the fractional populations 0.37, 0.36, 0.12, and 0.07, plus smaller contributions from another eight conformers that have a total contribution of 0.08. Clearly, there are two important conformers that are nearly equally populated, and the basic features of the final IR and VCD spectra are determined by these spectra. As a result of this analysis, not only has VCD been able to confirm the AC of (+)-ibuprofen as *S*, but two dominant solution-state conformers have been identified, along with two additional less-populated conformers.

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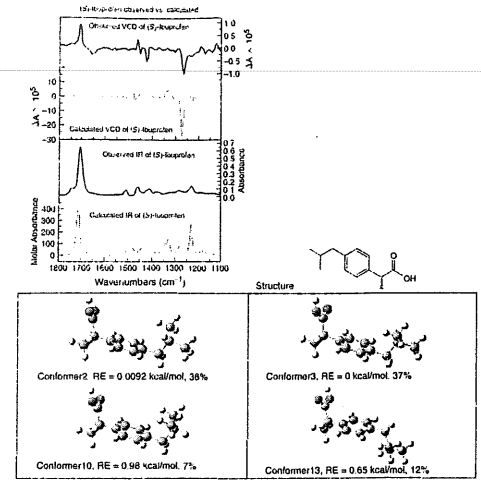

Figure 12. On the top are displayed the measured (observed; +) and calculated (*S*) IR and VCD spectra of (+)-ibuprofen (above) and the stereostructure of (*S*)-ibuprofen (below). On the bottom are displayed the stereostructures, relative energy (RE), and percentage Boltzmann population (BP) for the four most important solution-state conformers.

8. COMPARISON OF MEASURED AND CALCULATED SPECTRA

To provide an unbiased statistical measure of the degree of similarity between measured and calculated IR and VCD spectra, an analytical method based on a convolution algorithm has been developed. The degree of similarity, or congruence, between a measured and a calculated spectrum can be calculated and used to determine a degree of confidence that the AC determined by visual inspection is correct.

8.1 Degree of Confidence of Correct Assignment

The results of the use of such statistical measures are shown in *Figure 13*. The degree of similarity (the total neighborhood similarity, TNS) of the measured and calculated IR is 92.5, where 100 is a perfect match. The same TNS measure for the VCD is 82.2. Because VCD bands can be either positive or negative, a more refined analysis is carried out for VCD, and only regions of sign agreement are compared for each enantiomer. In this case, the value of the signed neighborhood similarity (SNS) for the *S*-enantiomer is 92.8, and for the *R*-enantiomer it is only 12.1. Clearly, the analysis confirms the visual agreement of the measured VCD spectrum (*upper solid line*) and the calculated VCD spectra (*lower solid line*) for the *S*-enantiomer versus the *R*-enantiomer (*dashed line*). An additional numerical comparison is calculated as the enantiomeric similarity index (ESI), which equals SNS(*S*)– SNS(*R*). The result (*black dot*) is then plotted against a database of 89 prior correct VCD assignments, where the axes are SNS (*vertical*) and ESI (*horizontal*). The closer a statistical point is to the *upper right-hand corner* of the plot, the higher the similarity of the comparison and the higher the degree of confidence. The upper right-hand corner of the plot is defined as 100% spectral similarity. The degree of confidence is a statistical measure that is set to be 100% confidence for points clustered in the *upper right-hand region* of the plot. Statistical measures such as these eliminate the reliance on only visual judgment for the comparison of measured and calculated VCD spectra and provide a statistical basis for assigning a degree of similarity between the measured and calculated spectra.

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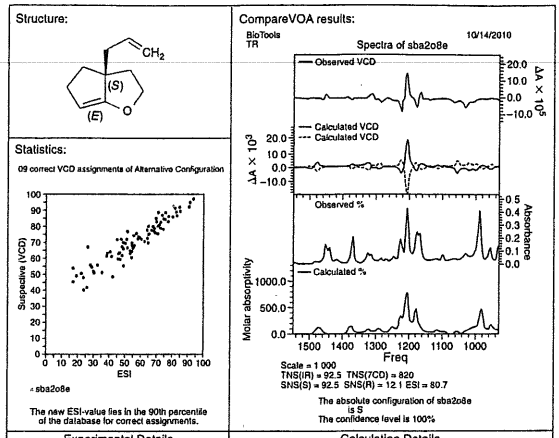

Experimental Details		Calculation Details	
Instrument	Chiral R2X with DualPEM	Gaussian Version	Gaussian 09
Solvent	CDCl3	DFT-level of theory	B3LYP/6-31G*
Acquisition time	10 h	Configuration calculated	As shown above
Concentration	7.5 mg/0.15 mL	Total calculated conformers	6
Pathlength	100 μm	Total low-energy conformer used for Boltzmann sum	4
Assigned Absolute Configuration: (*S*)-		Confidence level: 100%	

Figure 13. Output of a commercial program for comparison of measured (observed) VCD and IR spectra with calculated VCD spectra of both enantiomers and the calculated IR spectrum. Statistical data are explained in the text.

9. DETERMINATION OF ENANTIOMERIC EXCESS

After AC, the next most important property of a chiral sample is the EE, as described in *1. Introduction*. For enantiomers labeled *R* and *S*, the EE for the *R* enantiomer is defined as $EE = (N_R - N_S)/(N_R + N_S)$ and %EE = EE × 100%. Here, N_R represents the number of moles of the *R* enantiomer present in the sample; for a solution, N_R can represent the concentration of the *R* enantiomer. Thus, %EE for the *R* enantiomer can vary from +100% to −100%.

VCD spectra also can be used to determine the EE of a sample once the sample has been calibrated by a single IR and VCD measurement of a sample with a known EE. VCD scales linearly with EE with a maximum VCD intensity for a given IR intensity at 100% EE, one-half VCD intensity for the same IR intensity at 50% EE, and zero VCD for the racemic mixture of 0% EE. These points are illustrated in *Figure 14* for 11 measurements of *R*-(+)-α-pinene for which the %EE decreases from 100% to 6.7%. The IR spectra are identical, but the VCD spectra grow smaller linearly as EE is reduced by the addition of measured amounts of the opposite enantiomer to the sample cell. The degree of accuracy of this determination of %EE was slightly >1%, as indicated in the plot of actual prepared EE versus VCD predicted EE, using a partial least-squares chemometric analysis as presented in *Figure 15*.

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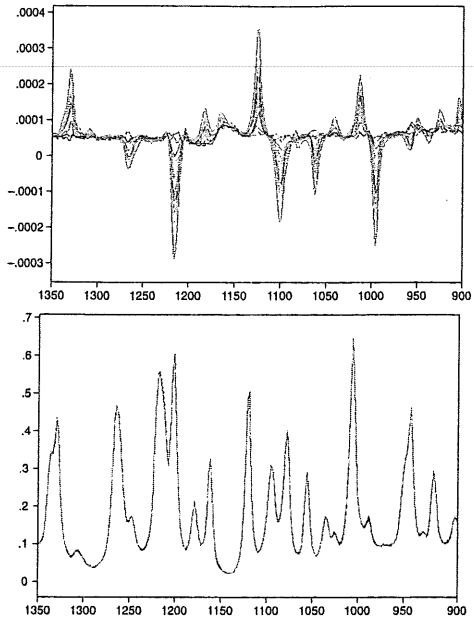
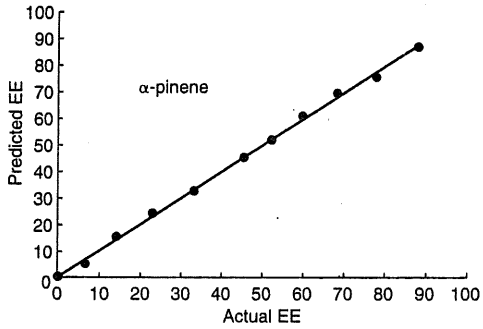


Figure 14. Superposition of 11 VCD (upper) and IR (lower) spectra of a 3.1 M solution of (+)-(*R*)-α-pinene in carbon tetrachloride for samples with %EE values of 100.0%, 88.2%, 77.8%, 68.4%, 60.2%, 52.4%, 45.5%, 33.3%, 23.1%, 14.3%, and 6.7%. Reproduced in part with permission from John Wiley & Sons.



Actual EE%	*Predicted EE%*	*Variance (EE%)*
88.2	87.2	1.0
77.8	75.9	1.9
68.4	69.7	−1.3
60	61.3	−1.3
52.4	52.2	0.2
45.5	45.3	0.2
33.3	32.8	0.5
23.1	24.4	−1.3
14.3	15.4	−1.1
6.7	4.9	1.8
0	0.3	−0.3
RMSD	1.15	
STDEV	1.21	

Figure 15. Plot and corresponding table of actual versus VCD predicted values of %EE using partial least-squares (PLS) chemometric analysis that achieves a root-mean-square deviation (RMSD) of 1.15 and a standard deviation error of cross-validation (STDEV) of 1.21.

Change to read:

10. CONCURRENT USE OF VCD FOR ABSOLUTE CONFIGURATION AND EE

10.1 Chiral Raw Material Identification

Currently, there is no routine, real-time chiral measurement for material identification (ID). Chiral drug substances must comply with the *USP–NF* monograph tests that typically rely upon nonchiral test procedures such as mid-IR spectroscopy (*Mid-Infrared Spectroscopy* ⟨854⟩ and *Mid-Infrared Spectroscopy—Theory and Practice* ⟨1854⟩). In practice, near-IR (for additional information, see ▲*Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩▲ (CN 1-May-2020)) is used in many manufacturing facilities. For near-IR analysis of solids, analysts commonly use fiber-optic probes and then confirm the raw material by comparison of the measured spectrum against that of the USP Reference Standard. To determine the AC and EE of a chiral material, analysts must make a separate optical rotation measurement, which requires large amounts of sample in a 10-cm path-length cell, where uncertainty errors are possible for materials with small OR values.

A single measurement of the VCD spectrum and its associated IR spectrum in the mid-IR or near-IR region of a raw material, either as a crystalline solid or in solution, simultaneously contains information for the three critical measures: ID, AC, and EE. By comparison to a USP Reference Standard, the simultaneous measurement of IR and VCD spectra of a test material identifies the sample and the presence of impurities and separates impurities into achiral (IR only) or chiral (IR and VCD). The signs of

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the VCD identify the AC of the dominant enantiomer, and the ratio of the VCD to the IR gives the EE, as described above. Typical VCD accuracy for EE determinations is in the range of 0.1%–1%.

10.2 Chiral Quality Control

VCD can be used as a chiral measure for characterization of raw materials for process analytical technology (PAT) during development, synthesis, formulation, and final production of drug substances and drug products. VCD also can be used to test the interaction between formulated chiral drug substances and excipients. Currently, there are no protocols for monitoring EE as a quality control measure in the pharmaceutical industry. Because of the importance of chirality as a critical measure of sample integrity, there is a need for the incorporation of a new technology, such as VCD, to ensure the desired level of chiral quality of pharmaceutical products from discovery through to final formulation.

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⟨1787⟩ MEASUREMENT OF SUBVISIBLE PARTICULATE MATTER IN THERAPEUTIC PROTEIN INJECTIONS

INTRODUCTION

Parenteral products are designed and manufactured to minimize particulate matter, which is differentiated into two broad categories: visible and subvisible. The absolute limit of visibility, or detectability, depends on the test conditions and the nature of the particulate matter. This general information chapter covers subvisible particles in the range of 2–100 µm. Particles larger than 100 µm are generally considered visible particles (see *Visible Particulates in Injections* ⟨790⟩).

Foreign and particulate matter are undesirable in the final product. These undesired particles arise from two sources: extrinsic or foreign matter and intrinsic or product-related matter. Extrinsic matter cannot be associated with product or process. Intrinsic particles may be a byproduct of the assembly of the product or result from change over time. A third category, inherent matter, describes a physical state or particles that are an expected attribute of the product. The aim of this chapter is to provide additional information to support *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, which has limits for ≤10-µm and

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≤25-µm particle content. Because the monitoring of the sub-10-µm population may be an important product quality parameter, collection of data in the 2–10-µm range (e.g. 2–5 µm and 5–10 µm) is recommended. This chapter will focus on enumeration, characterization, and when possible, identification of inherent particles, distinguishing them from extrinsic and intrinsic particles. The chapter does not cover formulations that are suspensions or emulsions, or those that contain adjuvants or similar intended particle components. However, foreign and particulate matter should be minimized in the final drug product, and the strategy and methods utilized are ultimately determined by the manufacturer.

Extrinsic Particles

Materials that are not part of the formulation, package, or assembly process, but rather are foreign and unexpected. Examples of extrinsic particles include materials from biological sources (e.g., fibers, insect parts, pollens, and vegetative matter), from process and building materials (e.g., cellulose, lint, minerals, glass, plastics, rubber, metal, and paint), and from personnel (e.g., epithelial cells, clothing fragments, and hairs).

Intrinsic Particles

Materials occurring in the final product that arise from sources within the formulation ingredients, assembly process, or packaging. Their association with the production process and/or package-related sources can be indicative of systemic problems. For example, silicone oil is an important manufacturing and product component that may affect particle counts and, in excess, is considered intrinsic. The incidence of silicone oil-related particles may correlate with processing sequences or formulation component sources. Detection, identification, and measurement of silicone oil-related particles are discussed further in another section of this chapter. Intrinsic particles can be similar in some ways to extrinsic particles such as rubber, metal, plastic, and glass, but may arise because of insufficient cleaning of processing equipment. Intrinsic particles can also arise from changes in the product over time. These changes may be related to ionic or organic extracts, instability of the active pharmaceutical ingredient (API), formulation degradation, or product–package interaction. Intrinsic particles may promote protein, and one should be aware of this possibility.

Inherent Particles

Materials that are expected from the drug substance and other formulation components, and thus represent a potentially acceptable characteristic of the product. In the context of therapeutic protein injections, the primary source of inherent particles is proteinaceous aggregates formed either solely by interactions of the protein with itself or in combination with other formulation ingredients. The presence of proteinaceous particles should be assessed for process consistency and product quality. Identification and characterization of the aggregates are critical for distinguishing them from extrinsic and intrinsic particles. The tools and techniques discussed in this chapter are applicable to all sources but are especially useful for the characterization of protein aggregates.

Heterogeneous particles consist of more than one chemical entity. They are classified as either extrinsic or intrinsic based on the nonproteinaceous component that presents the greatest risk.

Historically, test methods contained in *Particulate Matter in Injections* ⟨788⟩ were developed for detection and control of extrinsic and intrinsic particle content and rely on light obscuration (LO) and/or microscopic determination of particles that are between 10- and 25-µm thresholds. These sizes were selected to allow consistent measurement of subvisible particle content and allow determination of product acceptability from the perspective of patient safety and process consistency. The membrane microscopic method was developed first and was used only for large-volume (>100 mL) products. Later, improvements in the microscopic method and development of an automated light extinction (obscuration) method provided an efficient and robust means of tracking particulate content in a variety of products with different fill volumes. Evolution of analytical application is typical of the compendial standard-setting process, where methods are updated to reflect technological advances, patient safety considerations, and regulatory requirements.

Since ⟨788⟩ became official, the varieties of dosage forms and the numbers of therapeutic protein products have increased markedly. The LO method described in ⟨788⟩ has some technical limitations when used for analyzing certain particle types (e.g., those that have low contrast in the product medium and/or may change shape/size during analysis, as is typical for inherent particles in therapeutic protein injections). Other ⟨788⟩ limitations for therapeutic protein products relate to sample handling and low product volumes. These considerations have led to the development of *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩.

OBJECTIVE

This chapter was written to describe strategies for identifying and characterizing extrinsic and intrinsic particle populations, compared with inherent proteinaceous particle population(s), in therapeutic protein injections. Information about specific methods that can be used for these purposes, as well as their advantages and limitations, is discussed; the information applies to therapeutic protein injection products and their dilution or infusion solutions. The overarching goal is to provide comprehensive guidance on use of a broad array of analytical methods, one or more of which may be used beyond the methods described in ⟨787⟩ and ⟨788⟩. This is expected to achieve enhanced or orthogonal characterization of therapeutic protein products during development and support, root cause analysis for nonconformance investigations, stability studies, and others. This includes methods that allow assessment of a variety of characteristics of the inherent protein aggregates including morphology, conformation, reversibility/dissociation, and covalent modification. Guidance on sample handling and preparation is discussed in general terms.

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BACKGROUND

Extrinsic and intrinsic particles should be minimized in all parenteral products. However, protein-related particles may be inherent to therapeutic protein products. Inherent particles should be understood and controlled. These inherent particles are known and expected, arising from the association of protein molecules that can be present in a continuum of sizes, ranging from nanometers (dimers) to hundreds of micrometers (multimers and visible particles). The distribution of particle size is affected by amino acid sequence, solution conditions, sample handling history, and other factors. The specific phenomenon of protein association that gives rise to particles is called aggregation. The terms "proteinaceous particles" and "aggregates" are used interchangeably in this chapter.

Because multiple potential sources of particles exist, it is important to identify the particles and determine whether they are extrinsic, intrinsic, or inherent. Once this has been accomplished, it is possible to develop and apply appropriate control strategies. If deviations occur, particle identity will guide the root cause analysis, risk assessment, corrective actions, and control strategy.

Protein aggregates inherent in therapeutic protein products may consist of a heterogeneous population, and therefore size alone does not adequately describe them. When aggregate populations are being compared across products, labs, etc., it is important to consider other characteristics such as morphology, the ability to dissociate, the protein conformations (or higher-order structures) within the aggregates, and the chemical modifications present (*1*). These are summarized in *Table 1* [adapted from (*1*)]. It is important to realize that the results obtained depend on the technique and methodology used, and this information must be included in any description of protein aggregate characterization. The tools available for aggregate characterization are described later in this chapter. The tools used and the characteristics analyzed should be thoughtfully chosen based on the protein being studied. A more detailed description of attributes that should be considered during aggregate characterization follows.

Size is the particle attribute that has been monitored historically and remains a primary descriptor. When measurements are reported, they should include the size range measured and technique used. Thus, subvisible particles could also be described as aggregates/particles between 1 and 100 µm based on: the equivalent circular diameter, determined using LO; the longest chord, using light microscopy; the equivalent spherical volume, using electrical zone sensing; or other relevant dimensions obtained using other available technologies. The sample preparation, determination method, and algorithms applied are all important factors to include when presenting the results.

The ability of any aggregate species to dissociate (i.e., revert to the monomeric state) and the conditions required to accomplish this are also important characteristics to consider. Sample preparation could affect the results obtained if the aggregates are dissociable; therefore, understanding this aspect of the aggregates is critical when selecting techniques for counting, measuring, and characterizing them. The dissociability of protein aggregates can be assessed by diluting the aggregates into the formulation buffer or other solutions and then re-analyzing them with the original technique. This can also include simply returning the sample to the original conditions and assessing how much of the aggregate has dissociated. Ability to dissociate is thus a characteristic of protein aggregates that can inform sample handling and preparation (e.g., dilution conditions) for particle counting. The ability to dissociate may also apply to intrinsic and, to a lesser extent, extrinsic particles species.

The structure and conformation of protein molecules in the aggregates can be investigated by using several biophysical techniques. The findings may help in investigations and troubleshooting. It is also useful to know about the presence of chemical modifications such as oxidation, deamidation, cross-links, or fragments in the aggregate. Conformation of protein molecules within protein aggregates, as well as their state of covalent modification, may help explain the potential biological impact of the inherent particle.

Morphology of the particles can serve as another descriptor and also help identify their source. More specifically, characterization of morphology may allow one to distinguish between particles that are inherent to the therapeutic protein product and those that are extrinsic or intrinsic. Proteinaceous as well as many intrinsic and extrinsic particles are generally of irregular shape, whereas silicone oil and air bubbles tend to be spherical. The morphology of protein aggregates also constitutes important data that can facilitate comparisons across studies and over time.

Table 1 provides an overview of protein aggregate characteristics to consider, which can be used to classify them.

Table 1. Description of Categories of Protein Aggregates (*1*)

Category	Classification
Size	<100 nm (nanometer) 100–1000 nm (submicrometer) 1–100 µm (subvisible) >100 µm (visible)
Dissociability	Reversible (revert to initial state when returned to original conditions) Irreversible (do not dissociate under conditions tested) Dissociable (dissociate under specific conditions tested, e.g., dilution, changes in buffer, etc.) Dissociable under physiological conditions
Reversibility	Reversible Irreversible Dissociable Dissociable under physiological conditions Dissociable under defined (list) conditions
Secondary/tertiary structure	Native Partially unfolded Unfolded Inherently disordered Ordered (e.g., amyloid)

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Table 1. Description of Categories of Protein Aggregates (*1*) *(continued)*

Category	Classification
Covalent modification	Cross-linked (reducible and nonreducible) Intramolecular modification Oxidation Deamidation Fragmentation No modification
Morphology	Number of monomeric subunits Aspect ratio Surface roughness Internal morphology Optical properties Heterogeneous (e.g., protein–silicone oil)

Silicone Oil

A standard lubricant in pharmaceutical products and packaging. In excess, it may contribute to elevated particle counts in products. Assessing the effect of silicone oil on particle counts in therapeutic protein injections, where relevant, is critical to the overall particle control strategy. In parenteral manufacturing, silicone oil is used primarily to facilitate packaging component handling, alleviate adherence of rubber parts, allow free stopper flow in the bowl/hopper, and promote tracking onto the waiting, filled container. Silicone oil is also used as a lubricant to decrease glide force and allow plunger stopper movement in glass, pre-filled syringe presentations. For parenteral container preparation, controlled stages of washing, rinsing, siliconization, and sterilization are routine. The tumbling of closures (e.g., vial stoppers) with a measured amount of silicone has been a routine process for uniformly coating surfaces before use. Application of a known amount of an accepted lubricant, rendered sterile, is common.

Even when applied judiciously, silicone oil can migrate from the product-contact surface to the fill-over time during product transport, storage, and use. Silicone oil droplets can contribute significantly to the subvisible particle counts of the product, specifically, particles in the <10-µm range in the case of therapeutic protein injections. The particle count depends on the amount of silicone oil, and in some cases small amounts of silicone oil can have a significant effect on particle counts (*2*).

Within therapeutic protein products, protein may become adsorbed to silicone oil droplets. Agitation resuspends and rearranges the oily matrix into different particle forms and sizes. The size range of the silicone oil droplets in protein formulations may be similar to inherent protein particles, but the morphology and optical properties of the two substances differ. Silicone oil in aqueous solution is typically spherical, with a higher refractive index than that of protein aggregates and a regular decrease in contrast from the outside to the center of the particle. This is unlike protein aggregates, which are typically amorphous and translucent and have a refractive index very similar to the protein monomer background of the drug formulation. These distinctions can be used to develop algorithms for differentiating silicone oil particles from protein particles. It is also possible to monitor changes in one population and distinguish them from changes in the other.

Because silicone oil serves a function in the product, particle counts arising from the oil are considered intrinsic and generally will vary over time. However, poorly controlled application processes can lead to excessive amounts of silicone oil. Careful design, control, and application of the silicone oil are recommended to obtain proper functionality with the minimum amount needed for the shelf life of the product.

PARTICLE STANDARDS

Analysis of particle size distribution depends on the instrument used and its method of calibration. Some available particle standards (count and size) are polystyrene latex beads, mono-disperse silica or polymethylmethacrylate (PMMA) beads, and polydisperse glass beads. All of these standards are spheres, with a refractive index and density that are distinctly different from those of therapeutic protein products. Because typical protein particles have irregular morphology and low optical contrast, there are currently no standards available to consistently mimic the morphology and parameters of protein particles.

Protein particles have a refractive index that is quite close to that of the matrix solution. This difference in refractive index is a critical parameter for detection of protein particles by methods that rely on light interaction to measure size distribution. It is important to establish standard reference materials that mimic the properties of protein particles (*3*). The development of standards that better resemble protein particles is currently an active area of research. These standards should serve as surrogates for the actual protein particles in that the surrogate's properties mimic those of actual proteins for the detection method of choice. This approach is necessary because standards using proteins are limited by the following factors: (a) storage and transport should be at temperatures close to –80° to achieve acceptable stability; (b) any non-ambient technique may affect the standard; and (c) protein particles are themselves quite variable, making it difficult to have a single protein that would match all applications.

SUBVISIBLE PARTICLE MEASUREMENT AND CHARACTERIZATION TECHNOLOGIES

The following sections review the advantages, limitations, and appropriate uses of each methodology (see *Table 2*) that allow assessment of a variety of characteristics of all particles, especially for the inherent protein aggregates, including size and distribution, size and morphology, and characterization. The methodology selected will affect reported particle size because of instrumental measurement principles, mode of measurement, particle characteristics, preparation, and handling. It can also be affected by the algorithms applied to estimate size, volume, and count. Thus with any size measurement, the method used should be specified. Also, the units should be clearly stated, because size can be provided using various parameters, usually

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hydrodynamic diameter, equivalent circular diameter (ECD), longest chord, equivalent spherical diameter (ESC), Feret's diameter, or molecular weight. The list in *Table 2* is not meant to be all inclusive.

Table 2. Methodologies Useful in Measuring Properties of Subvisible Particles

Section I: Size and Distribution		
Technique	**Principle of Operation**	**Range**
Light obscuration	The size of the particle in the product fluid is determined by the amount of light that it blocks when passing between the source and the detector.	1–300 µm
Electrical sensing zone (Coulter)	The size of the particle in the product fluid or selected electrolyte is measured in terms of the change in resistance as the particle passes through a microchannel (orifice).	0.4–1600 µm
Laser diffraction	The size of the particles in product fluid or dilution is determined by measuring the angle of the scattered light.	0.1–3500 µm
Section II: Size and Morphology		
Technique	**Principle of Operation**	**Range**
Light microscopy	Photon imaging of substances directly in product fluids or mounts, or of isolated specimens on substrates	0.3 µm to 1 mm
Flow imaging analysis	Digital image capture of the particles' magnified image in streaming product fluid, revealing size, shape, and optical properties	0.7–100 µm for size distribution; 4–100 µm for morphology
Electron microscopy (EM): Scanning EM, scanning transmission EM, and transmission EM	Electron imaging of specimen isolates on substrates. High vacuum or near-ambient pressures is required.	Å to mm
Section III: Characterization		
Technique	**Principle of Operation**	**Range**
Fourier Transform Infrared (FTIR) microspectroscopy	Photon imaging of isolated specimens on substrates	10 µm to 1 mm
Dispersive-Raman microspectroscopy	Photon imaging of isolated specimens on substrates, or in product fluids or fluid mounts	0.5 µm to 1 mm
Electron microscopy (EM) with energy-dispersive X-ray spectrometry (EDS)	X-ray photon emission from specimens energized by a focused electron beam	Å to mm for imaging; 1 µm to 1 mm for elemental composition
Electron microscopy (EM) with electron energy loss spectroscopy (EELS)	Inelastic scattering from specimens energized by a focused e-beam; e-loss is characteristic of the source element; complementary to EDS	Å to mm for imaging; 0.5 µm to 1 mm for elemental composition
Time-of-Flight Secondary Ion Mass Spectrometer (TOF-SIMS)	Identification of particles according to their mass spectra profile	µm to near mm
Staining assay	Visible staining to gain qualitative confirmation of unknown materials	0.3 µm to 1 mm

The accuracy of equipment used to determine particle size distribution is verified by calibration with reference particles of known concentration and size traceable to the International System of Units.

The concept of three-dimensional particle size is critical to understanding how particle standards interact with the various types of measurement equipment and how to compare the results across methodologies. Different techniques use different measurements and algorithms to determine size (e.g., volume equivalent diameter, surface equivalent diameter, and drag or Stokes diameter).

SIZE AND COUNT DISTRIBUTION

Light Obscuration (working range 1–300 µm)

PRINCIPLE OF OPERATION

The sample is passed between a light source and a sensor. Counts are generated when individual particles pass between the two, disrupting the light, which yields a voltage spike. The height of each voltage spike depends on the size of the particle causing it. The particle size to be recorded is generated from a size–voltage response calibration curve constructed using monosphere, certified reference size standards (typically polystyrene beads). LO tabulates particle size as the diameter of a circle having an equivalent cross-section. The product may be sampled directly from the container, pooled from several containers, or prepared as a dilution (see general chapters *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩, ⟨788⟩, *Particulate*

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Matter in Ophthalmic Solutions ⟨789⟩, and *Methods for the Determination of Particulate Matter in Injections and Ophthalmic Solutions* ⟨1788⟩).

ADVANTAGES

- Primary *USP–NF* method with large archive of historical data
- Particles directly observed in solution
- Measurements are straightforward and quick
- Capable of unit–unit sampling; analyzes almost entire sample
- Easily implemented
- Medium-to-high sample throughput.

LIMITATIONS

- One sensor head cannot cover the full working range
- Tabulates particle size as the diameter of a circle having an equivalent cross-section
- Dilution of the sample may be required and may change sample properties
- Upper limits for particle load that can be measured depend on sensor used and potential for coincidence counting
- May underestimate subvisible protein particles formed in formulation because of low contrast
- Medium should be transparent for laser light
- Sensitive to air bubbles, and some degassing techniques can change sample properties
- Destructive, i.e., sample cannot be reused.

Electrical Sensing Zone (Coulter Principle) (working range 0.4–1600 µm)

PRINCIPLE OF OPERATION

The sample is diluted into an electrolyte solution and drawn through a small aperture, passing between active electrodes, interrupting an electric field. Response is based on the displacement volume of the electrolyte, and thus size is reported as the equivalent spherical diameter (the diameter of a calibration standard sphere of the same volume as the particle). The response is unaffected by particle type (e.g., variations in color, hardness, opacity, refractive index).

ADVANTAGES

- Determines particle size in terms of equivalent spherical volume
- Not affected by optical properties of the solution
- Orthogonal technique to light-based methods, i.e., LO
- Medium-to-high sample throughput for a single orifice.

LIMITATIONS

- Low particle concentration required
- Each orifice has limited measuring range (2%–60% of orifice diameter)
- Near the lower end of the working range, electrical noise may be hard to distinguish from true particle counts
- For broad particle size distribution, more than one orifice may be necessary, which will involve reduced sample throughput
- Sample must be diluted in electrolyte solution if the formulation buffer does not have sufficient conductivity; the dilution may alter sample properties
- Smaller orifices require higher conductivity of medium and can require significantly increased buffer concentration
- Destructive, i.e., sample cannot be reused.

Laser Diffraction (working range 0.1–3500 µm)

PRINCIPLE OF OPERATION

Laser diffraction detects particles passing through a laser beam as they scatter light at an angular distribution of intensity that depends on the size of the particles. Therefore, it is possible to calculate particle size distributions if the intensity of light scattered from a sample is measured as a function of angle. This angular information needs to be compared with a scattering model (Mie theory) to calculate the size distribution.

ADVANTAGES

- Brief analysis time (up to 30 s)
- Analytical samples can be retrieved
- Limited sample preparation
- Scattering pattern allows molecular mass calculation

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- High sample throughput
- Large dynamic range.

LIMITATIONS

- Intensity is directly proportional to size, which can lead to misinterpretation of the population
- Analysis relies on a theoretical fit that requires refractive index and other optical factors
- Medium should be transparent for laser light
- Assumes particles are spherical and not interacting with each other
- Low concentration of particles required; dilution may be necessary and may change sample properties
- Complications can occur when comparing data from one instrument vendor to another
- Particle–particle interactions and occasionally multiple scattering may yield apparent size results that differ significantly from actual particle size
- Poor results for polydispersions when small and large particles are presented simultaneously
- For exact molecular weight calculation, monodisperse particles are required.

SIZE AND MORPHOLOGY

Light Microscopy (LM) (working range 0.3 µm to 1 mm)

PRINCIPLE OF OPERATION

Optical or light microscopy involves passing light through a series of lenses after it is transmitted through or reflected from the prepared sample. The light is detected directly by the eye, imaged on a photographic plate, or captured digitally. Samples can be analyzed directly or after filter capture. Light microscopy can be combined with static imaging analysis, thereby allowing digital images to be captured and further deconvolved using the software systems in image analysis. When using image analysis, one may select certain parameters to screen out unwanted artifacts or particle populations (circularity, aspect ratio, and others) and then begin studying the selected population set (see *Optical Microscopy* ⟨776⟩, ⟨788⟩, ⟨789⟩, and ⟨1788⟩).

ADVANTAGES

- Direct observation of particles in product fluid and filter capture
- Visualization, counting, sizing, and morphological characterization of particles are readily obtained
- Direct observation of particle may provide immediate recognition and identification
- Can be coupled with spectroscopic analyses (infrared, Raman) to identify chemical composition
- Particle identification can be enhanced via image database search and comparison
- Only small sample volume is needed for direct observation
- Membrane filtration is secondary to the ⟨788⟩ LO method.

LIMITATIONS

- Time consuming
- Low sample throughput
- Limited depth of field at high magnification can affect sizing accuracy
- Only a small sample volume is analyzed, so sampling must be done carefully
- For filter-captured samples, protein particles can pass through membranes and may present as amorphous background
- For filter-captured samples, potential exists for sample preparation artifacts
- Difficult to visualize particles with low optical contrast; must use phase, differential interference contrast microscopy, polarized light microscopy, and other methods
- Destructive, i.e., sample cannot be reused for filter-captured samples.

Flow Imaging Analysis [working range 0.7–100 µm (size) and 4–100 µm (morphology)]

PRINCIPLE OF OPERATION

This method captures digital images of materials within a flowing sample stream and uses post-collection analyses of the stored images. As in optical microscopy, which is essentially a static image analysis system, dynamic image analysis or flow microscopy captures multiple separate images, in this case using a dynamic, flowing system. Dynamic image analysis uses the components of illumination, objective lens, and focusing lens of a microscope, and adds a closed fluid pathway within a defined dimensional flow cell, plus camera and processor for acquisition and analysis. Thus, the method provides in situ conditions with realistic views of the particles. Visualization of the particles of gas, liquid, semisolids, or solids with this technique aids the interpretation of other in-suspension measurements that cannot visualize the particles being studied. The particles are moving and are likely tumbling, and this must be accounted for in the system design or data consideration. Increasing the system magnification and pixel resolution and reducing the flow cell depth will augment the quality of the captured image. An

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additional advantage of dynamic flow microscopy is the ability to apply size and feature analyses to the particle image. Also, any number of additional algorithms may be applied to study the collected image set as necessary. Particle image clarity, contrast, and shape may indicate possible source or type of particle, and with sufficient comparator data, mathematical filters can be applied to analyze and quantify different populations in a sample. This approach has been used to help distinguish silicone oil from protein aggregates.

ADVANTAGES

- Direct observation of particles in product fluid (in situ)
- Analysis of captured digital images allows visualization, counting, sizing, and characterization of particles
- Retained images may be further analyzed by different algorithms
- Actual particle images facilitate understanding of contamination sources and product nature
- Compared to LO or membrane microscopy (MM), increased sample volume and reduced analysis time
- Morphology and relative refractive index of particles apparent, easily calibrated
- Technique can be used with image databases to identify some particles
- High repeatability, high resolution, and good selectivity
- Medium-to-high sample throughput.

LIMITATIONS

- Minimum particle size necessary
- Results dependent on algorithm used to select, classify, and size particles
- Difficult to visualize and appropriately quantify number and size of particles with low optical contrast, such as translucent proteins
- Dilution of sample may be required and may change sample properties
- Destructive, i.e., sample cannot be reused.

Electron Microscopy (working range Angstroms to mm)

PRINCIPLE OF OPERATION

In EM, a beam of electrons is used to illuminate the samples and produce a magnified image. The practical spatial resolution limit of EM for biological samples is approximately 1 nm, which greatly exceeds resolution requirements for typical >0.1-µm particles in protein solutions. For EM analysis, subvisible particles should be either individually isolated or captured on a suitable filter and then mounted onto special grids or stabs for observation in the microscope. Traditional EM operates in high vacuum (about 10^{-6} Torr). Sample drying and coating is a required preparation step. Newer technologies allow examination of uncoated, as-is samples (environmental or wet EM). Detection techniques are divided as follows: (a) scanning electron microscopy (SEM), which may use secondary electron or back-scattering signals; (b) transmission electron microscopy (TEM), which visualizes electrons penetrated through sample; and (c) scanning tunneling electron microscopy (STEM), which combine SEM and TEM principles (see also *Scanning Electron Microscopy* ⟨1181⟩).

ADVANTAGES

- Visualization, counting, sizing, and morphological characterization of particles are readily obtained
- Direct observation of particle may provide immediate recognition and identification
- High repeatability, high resolution, and good selectivity
- Information about chemical composition of object can be obtained using energy-dispersive spectroscopy (EDS) or electron energy loss spectroscopy (EELS) (see below)
- Enhanced depth of field compared with photon methods
- Image analysis and building permits quasi-3D imaging of particles.

LIMITATIONS

- Time-consuming preparation and analysis
- Low sample throughput
- Possible changes in structure and size may arise from sample preparation and high-vacuum operation
- Specialized sample preparation requirements may exist, depending on the nature of the sample
- Contrast staining with heavy metals salts may be required for enhancing protein samples
- Protein particles are flexible and can pass through membranes
- Destructive, i.e., sample cannot be reused.

CHARACTERIZATION

Characterization of the protein active ingredient includes not only size distribution assessment but also determination of the chemical nature of the primary protein as well as conformation, aggregation, and self-associated species (see *Table 1*). The size

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of the fundamental particle–aggregate is important; however, the nature of the solids and the association with other species are similarly important.

Fourier-Transform Infrared (FTIR) Microspectroscopy (working range 10 µm to 1 mm or greater)

PRINCIPLE OF OPERATION

When light interacts with a substance, the light can be reflected, absorbed, or scattered. Infrared spectroscopy determines the vibrational properties of matter, typically in the 200–4000 cm^{-1} range, by analyzing the interaction with light while detecting the absorption of photons. In addition, the use of microscopy-based instruments, thus microspectroscopy, allows selection of individual particles or domains of interest, with the benefit of filters and polarizers for microscopes and the ability to capture an image, along with vibrational or other imaging data. Vibrational spectroscopy techniques are particularly useful for analysis of particles in protein solutions.

Particles can be isolated individually or captured on a membrane filter and then analyzed using FTIR microscopy. Because the infrared signal from water interferes strongly with protein bands, these samples usually require thorough drying before analysis. Typically, FTIR microscopes can operate in either image mode or spot mode. In image mode, the infrared signals are collected from individual pixels in the pre-defined area. However, spot mode usually provides higher signal-to-noise ratio, because the combined infrared signal is collected from the area defined by the aperture size. Many organic compounds have unique infrared signatures. Spectra collected for an individual particle can be analyzed by performing an infrared spectra library search or by comparing the particle's spectra results with those of suspected materials.

ADVANTAGES

- Microspectroscopy has the same sample advantages as LM
- FT instruments excel compared with dispersive spectrometers (grating monochromator) for particle analysis
- Provides rapid chemical identification and classification of particles, manually or automatically, in comparison with spectral databases
- Identification, structure, and composition can be derived from spectral data
- Provides some information on protein structure
- Nondestructive; samples can be reused.

LIMITATIONS

- May have the same limitations as LM
- FTIR provides strong absorption for common materials, such as silicones, water, and glass, which may interfere with or hinder sample analysis
- Identification depends on availability of information on possible components in spectral libraries
- Particle size limit is dependent on instrument resolution (~10 µm for FTIR)
- Process of particle identification becomes time consuming, if not available in a database
- FTIR is not sensitive to many inorganic materials or other molecules that do not possess a dipole moment
- Certain detectors are not sensitive to inorganic materials.

Dispersive-Raman Microspectroscopy (working range 0.5 µm and greater)

PRINCIPLE OF OPERATION

As with infrared spectroscopy, Raman spectroscopy is the study of how light interacts with a substance. A defined, monochromatic photon source (laser) is focused on the sample, thereby producing reflectance, absorption, and scatter, probing the same vibrational states probed by infrared microspectroscopy. Specifically, Raman spectroscopy is the study of the inelastically scattered photons (Raman scattering). Photons are also scattered elastically (Rayleigh scattered) with no change in wavelength, but are of use only to mark the laser excitation energy. A virtual excitation state is attained, which then relaxes to a base vibrational or rotational state, emitting energy characteristic of a functional group such as molecular and related atoms. Lower (Stokes) and higher (anti-Stokes) shifts are recorded due to interaction with the electron cloud of the functional group bonds. Lasers are used because only a very small proportion of Raman-scattered photons (only about 1 in 10^6–10^8) exhibit wavelength shift. Raman microspectroscopy combines a light microscope with a coincident laser and white light pathway. The microscope, with the benefit of filters and polarizers, aids in selection of individual particles or domains of interest to allow capturing of an image, along with vibrational spectra.

Fourier-Transform Raman is generally complementary to dispersive Raman, and in using longer wavelength excitation yields little sample fluorescence. One important advantage of Raman microscopy compared with infrared is the ability to analyze aqueous samples directly, often in glass sample holders. Individual particles can be analyzed in situ, and in some cases directly in the container, if it does not interfere with the Raman signal. Similar to the infrared analysis, unknown spectra can be analyzed either by a spectral library search or by matching the spectra of individual reference compounds.

Raman spectroscopy theory and techniques are further described in *Raman Spectroscopy* ⟨1120⟩.

ADVANTAGES

- Same sample advantages as LM

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- Dispersive instruments excel over Fourier-Transform spectrometers for particle analysis
- Rapid chemical identification and classification of particles, manually or automatically by comparison with spectral databases
- Identification, structure, and composition can be derived from spectral data
- Provides some information on protein structure
- Good lower size limit; instrument resolution is ~0.5 µm for dispersive-Raman technique
- Raman analysis viable for many organic and most inorganic compounds
- Identification uses Raman shift data, known for molecular categories and functional groups
- Nondestructive; samples can be reused
- Glass and plastic packaging have weak Raman spectra, thus interference from packaging is minimized.

LIMITATIONS

- Same limitations as with LM
- Specimen fluorescence may hinder data collection
- With sensitive samples, there can be laser-induced changes (heat, light)
- Many organic species yield no Raman shift; that is, there is no molecular polarization
- Identification may depend on the information available in proprietary and public spectral libraries.

Electron Microscopy (EM) with Energy-Dispersive X-Ray Spectrometry (EDS or EDX) (working range for qualitative analysis, 0.1- to 3-µm particles; for semiquantitative analysis, >3-µm particles)

PRINCIPLE OF OPERATION

Energy-dispersive X-ray spectrometry (EDS or EDX) is based on characteristic X-ray photon emission generated from a specimen through interaction with a high-energy source, such as an electron beam. Typically, an elemental intensity is plotted against X-ray emission energy, which correlates with the content of atomic valence shell transition energies. Current instrument technology uses thin-window and windowless detectors positioned near the sample in EM systems, which improves detection and signal-to-noise ratio for all elements, especially lighter elements, e.g., $Z < 11$ (sodium). Immediate, qualitative elemental composition is attained for solid specimens (see also ⟨1181⟩ for general EM and related EDS background information).

ADVANTAGES

- Complementary to EM and available for most commercial electron microscopes
- Determines elemental composition of the sample
  - Rapid qualitative collection for small particles
  - Semiquantitative analysis (1% LDL and ±20% RSD) possible in controlled experiments
- Most useful during survey investigation of particle origin
- Involves manual elemental screening and/or search of databases
- Elemental mapping can assist in protein particle visualization.

LIMITATIONS

- Not all instruments are equipped for determination of light elements (e.g., $Z < 11$)
- Quantitative analysis entails measuring line intensities for each element in the sample and for the same elements in calibration standards of known composition
- Strong background may be contributed by filter membrane material and sample coating.

Electron Microscopy (EM) with Electron Energy Loss Spectroscopy (EELS) (working range 0.5 µm to 1 mm)

OPERATION PRINCIPLE

Electron energy loss spectroscopy (EELS) measures the vibrational motion of atoms and molecules on and near the surface by analyzing the energy spectrum of low-energy electrons back-scattered from it.

ADVANTAGES

- Used in conjunction with transmission electron microscopy
- Determines elemental composition of the sample, especially light elements (e.g., $Z < 11$)
- Can provide information about chemical bonding
- Qualitative and quantitative analyses are possible
- Has lower detection limits than in EDS.

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LIMITATIONS

- Difficult to interpret
- Strong background
- Quantitative analysis entails measuring line intensities for each element in the sample and for the same elements in calibration standards of known composition.

Time-of-Flight Secondary Ion Mass Spectrometer (TOF-SIMS) (working range µm to near mm)

OPERATION PRINCIPLE

Time-of-Flight Secondary Ion Mass Spectrometer (TOF-SIMS) is a surface-sensitive analytical method that uses a pulsed ion beam [microfocused cesium (Cs) or gallium (Ga)] to remove molecules from the very outermost surface of the sample. The particles are removed from atomic monolayers on the surface (secondary ions). These particles are then accelerated into a "flight tube", and their mass is determined by measuring the exact time at which they reach the detector (i.e., time-of-flight).

ADVANTAGES

- Surveys of all masses on material surfaces; these may include single ions (positive or negative), individual isotopes, and molecular compounds
- Elemental and chemical mapping on a submicron scale
- High mass resolution, to distinguish species of similar nominal mass
- High sensitivity for trace elements or compounds, on the order of ppm to ppb for most species
- Surface analysis of insulating and conducting samples
- Depth profiling (in the near surface environment, on the order of individual atomic layers to 10 s of nanometers)
- Nondestructive analysis
- Retrospective analysis, for post-data acquisition analysis and interpretation of stored images and spectra

LIMITATIONS

- Generally does not produce quantitative analyses (semi-quantitative at best)
- Optical capabilities typically limited, making it difficult to find grains or specific regions of interest for analysis
- Charging may be a problem in some samples, although charge compensation routines are generally sufficient to overcome these problems
- There is commonly an image shift when changing from positive to negative ion data collection mode, making it difficult to collect positive and negative ion data on exactly the same spot.

Staining Assay (working range 0.3 µm to 1 mm)

OPERATION PRINCIPLE

Staining methods utilize reagents to provide specific reactions, such as visible staining or fluorescence, which provide qualitative confirmation of an unknown materials category or identity. Generally conducted via microscopical methods; microscopy advantages and limitations apply.

ADVANTAGES

- Direct observation of staining effects upon particles in product fluid and by filter capture
- Visualization, counting, sizing, and morphological characterization of particles are readily obtained
- Direct observation of particle may provide immediate recognition and identification
- Can be coupled with spectroscopic analyses (infrared, Raman) to identify chemical composition
- Surface analysis of insulating and conducting samples
- Particle identification can be enhanced via image database search and comparison
- Only small sample volume is needed for direct observation

LIMITATIONS

- Low sample throughput
- Limited depth of field at high magnification
- Only a small sample volume is analyzed, so sampling must be done carefully
- For filter-captured samples, protein particles can pass through membranes and may present as amorphous background
- For filter-captured samples, potential exists for sample preparation artifacts
- Difficult to visualize particles with low optical contrast; must use phase, differential interference contract microscopy, polarized light microscopy, and other methods
- Destructive; that is, sample cannot be reused for filter-captured samples

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STRATEGY

Particles in therapeutic protein products generally have continuous size distributions with exponentially higher numbers of the smaller (<10 µm) particles. Information generated from analysis of inherent protein particles can be used for guiding development efforts and to facilitate communication and understanding of this important quality attribute of therapeutic protein products. A rational strategy should be developed for characterizing protein aggregates or particles. The categories in *Table 1* provide a guideline for differences observed between types of protein aggregates, recognizing that size distribution analysis of these species is important, but it is not the only informative characteristic. Describing the characteristics of the protein aggregates in the manner shown in *Table 1* can help define the population and/or characteristics that may be the most important for assessment of the effect on product quality.

To obtain a comprehensive characterization of the particle distribution, a variety of analytical techniques must be applied. There are a number of techniques available to measure and characterize particles, each with its advantages and limitations. Multiple methods are needed because they differ in their ability to analyze specific attributes of the particle. By using orthogonal methods, one can better ascertain the nature of the particles (extrinsic, intrinsic, or inherent), the size distribution, and other aspects that may allow differentiation between particles. Information about the particle characteristics can be used to perform a risk assessment around the presence of aggregates and to help identify potential mechanisms of formation. This knowledge about the particles' characteristics can also be used during formulation and process development to inform choices of the formulation and process steps, which can help in reducing the overall particle content of the drug product.

A comprehensive strategy for assessing subvisible particles involves multiple phases from development through commercialization and during marketing. Particle measurement/characterization may begin as early as candidate selection, with the use of high-throughput techniques to predict particulate-forming tendencies of the molecules under consideration. This involves using small amounts of material and providing a relative ranking of aggregation propensity.

Early Development

At this stage, the focus is on understanding the typical particle profile for the product, including the size distribution and the characteristics of the aggregates most commonly seen in the drug product. The particle size and count in the 1- to 100-µm range should be monitored at the time of manufacture and during stability.

Late Development

At this stage, the focus is on understanding the drug product and comparability between lots, as well as the linkage between particle size analysis and formulation, manufacturing, and use. Particle size and count in the 1- to 100-µm range should be monitored in clinical batches. Enhanced characterization of aggregates and particles, including pivotal product batches, should be performed. Quantitative and qualitative data about the subvisible particles formed under storage, use, and stress conditions should be collected, and risk mitigation strategies created. When the final method for monitoring the product's particle size and count is chosen, control strategies for inherent and intrinsic particles should be established. The overall control strategy developed may include action limits beyond which an investigation may be warranted.

Marketing

At this stage, one should collect data over commercial batches to align with the overall control strategy, which may include the sub-10-µm (1- to 10-µm) range. Use quantitative as well as qualitative analysis during the management changes for the post-marketing life cycle. When an investigation occurs, the knowledge obtained during development can be used to design mitigation strategies. Stability studies are also part of life cycle management, and are addressed later in the *Application* section.

Overall Perspective

In-depth characterization of aggregates during development, coupled with correlation of results from these tests to those used for product release (such as LO), may provide the basis for a rational, overall control strategy. Aggregates are usually present in a continuum of size from oligomers to particles that are hundreds of micrometers. If a correlation (or mathematical relationship) can be demonstrated in the counts of particles from oligomers through the >25-µm sizes assessed as part of the compendial method, it might be possible to rely on the LO determination of the >10- and >25-µm particle content to reveal the effect of changes and trends in manufacturing on 1- to 10-µm particles. In this situation, the overall control strategy would be used to establish action limits for particles >10 µm, which when exceeded would trigger an investigation, including the characterization methods discussed in this chapter.

SAMPLE CONSIDERATIONS

Certain aspects of sample handling and volume require special consideration. Handling and degassing procedures need to be developed and applied to avoid artifacts such as false positives attributable to bubbles, or aggregates generated during the sample preparation steps. Under certain circumstances, dilution of samples may be necessary to obtain reliable results. Aspects of sample handling are described in ⟨787⟩.

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APPLICATION

A key focus during development of a protein drug product is to gain knowledge about the nature, source, and number of inherent particles/aggregates present in the therapeutic formula, as well as their stability and overall effects on product quality, safety, and efficacy.

Protein aggregates in the 1- to 10-µm size range may also carry a risk of potentiating immunogenicity against the therapeutic activity (*4,5*). From a product-development perspective, changes in the concentrations of proteinaceous subvisible particles, particularly in the 1- to 10-µm range, can be an early and sensitive indicator of potential issues with product stability (*6,7*). Apart from stability, particles in these size ranges should also be measured as part of the risk assessment exercise to improve understanding of how various stressors and conditions of use affect the product. These conditions could include thermal, freeze/thaw, light, transport, dilution/admixture, and use characteristics. Particle counts in the 1- to 10-µm range tend to be significantly (orders of magnitude) greater than those in the ≥10-µm range. This makes the 1- to 10-µm range useful for the purpose described above, because changes can be easier to observe. However, the counts also tend to be quite variable, and thus the ability to discern trends requires proper controls, adequate sampling, and good technique.

Detailed characterization of subvisible particles, especially inherent particles, provides information that can support the formulation and product development program, leading to a robust process and commercial control strategy. On the other hand, although characterization alone may not reveal possible correlations between an immune response and aggregates/particles, the use of multiple methods applied to multiple drug product lots helps inform the definition of product quality, which can be linked to safety and efficacy data obtained from clinical trials. This knowledge is also useful in life cycle management, such as movement of product between manufacturing sites, changes in presentation (liquid to lyophilized; vial to prefilled syringe), and changes in strength.

To illustrate these points, two examples are presented below; they show ways that complementary techniques can be used to gain product knowledge.

Example 1: Comparison of Drug Product Configurations

Not all drug products are formulated as liquids. In one case of a lyophilized drug product filled in glass vials, a liquid formulation in a prefilled syringe configuration was developed to facilitate patient administration. A comparison of subvisible particle counts by LO was expected to show that the particle count of the reconstituted lyophilized formulation was higher than in the liquid formulation, but the opposite results were observed, with the liquid formulation containing significantly higher levels of particles >2 µm [~130,000 particles per container (ppc) compared to ~30,000 ppc]. These samples were then assessed by the MM method, and the results were more consistent with the expected outcome: for particle sizes >5 µm, the lyophilized formulation contained ~70 ppc compared to ~14 ppc for the liquid formulation. Analysis was then performed using flow microscopy to understand the discrepancy in results between the MM and LO methods. Flow microscopy supported the LO results, with the liquid formulation showing higher numbers of particles >2 µm (~600,000 ppc) compared to the lyophilized formulation (~150,000 ppc). Examination of the structure of the particles revealed a different morphology in the liquid formulation, with a predominant number of the particles exhibiting a spherical shape, consistent with the presence of silicone oil droplets from the prefilled syringe container. These results were confirmed by testing a sample of the liquid formulation that had not been exposed to the prefilled syringe (the source of the silicone oil), which showed <20,000 ppc. Once the silicone oil droplets were accounted for, the number of remaining particles in the liquid formulation in the prefilled syringe was lower than in the lyophilized formulation, consistent with the MM results.

Example 2: Selection of Filling Technology

Filling processes can affect product quality by introducing physical stresses such as adsorption, shear, friction, and cavitation, which may lead to protein aggregation. Certain drug product filling pumps may shed extrinsic particles that can lead to heterogeneous nucleation-induced aggregation.

The effect of using a stainless steel displacement piston pump on the subvisible particulate content of solutions was investigated using multiple techniques to analyze the particulates in solution (*8*). Coulter counter analysis of a buffer solution passed through the pump revealed that the pump shed particles in solution, with the pumped solution containing >13,000 particles per mL in the 1.5- to 6-µm range. Elemental analysis confirmed that the particles were stainless steel. Analysis of the particle size distribution in this solution, performed using a laser diffraction particle sizer, showed that most of the particles were between 0.25 and 0.95 µm. Incubation of stainless steel nanoparticles with a solution of MAb-Y led to increases in both the number of particles and the particle size distribution over time. Characterization of the particles by FTIR spectroscopy showed that the protein adsorbed onto the metal particles contained a slightly perturbed secondary structure compared to the protein in solution. It was concluded that the metal particles served as nucleation sites for protein aggregation. In this case, selection of a different type of filler may be warranted to mitigate protein aggregation.

A comparison of the effect of different pump types on subvisible particulate formation in a solution of MAb-Z was performed (*9*). Flow microscopy showed that piston pumps, made of either stainless steel or ceramic, produced particles in the size range of 1–100 µm at levels approximately 100-fold higher than the control samples. Examination of the microscopic images of these particles revealed that the particles were translucent, suggesting they were protein based. Incubation of the protein solution with shed particles from the piston pumps did not lead to a significant increase in either the particle size distribution or particle counts for these solutions, indicating that extrinsic particles did not serve as nucleation sites for the protein. In this case, the formation of particles in the solution was considered to be from shear stress caused during the pumping operation. These results were supported by examination of other filler types (peristaltic, time-pressure, and rolling diaphragm), which produced solutions with significantly lower particle load.

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SUMMARY

This chapter provides strategies for describing, characterizing, and identifying the particle content in therapeutic protein injections, including extrinsic and intrinsic particle population. Information regarding specific methods that can be used for these purposes, as well as their advantages and limitations, is discussed. A rational, comprehensive control strategy can be developed on the basis of an in-depth characterization of aggregates during development, coupled with correlation of results from these tests to those used for release. Aggregates are usually present in a continuum of sizes, from oligomers to particles that are hundreds of micrometers. Correlation in the counts of particles from oligomers through the >25-µm size, assessed as part of the compendial method, may make it possible to rely on the LO determination alone to reveal changes and trends in manufacturing. In this situation, the overall control strategy would be used to establish action limits for particles >10 µm, which when exceeded would trigger an investigation, including the characterization methods discussed in this chapter.

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⟨1788⟩ METHODS FOR THE DETERMINATION OF PARTICULATE MATTER IN INJECTIONS AND OPHTHALMIC SOLUTIONS

INTRODUCTION

It is the intent of USP general test chapters *Injections and Implanted Drug Products* ⟨1⟩ and *Particulate Matter in Injections* ⟨788⟩ that systems, packaging, and formulation be selected and developed (or refined) with regard to commercial product cleanliness and stability. Process streams, equipment, filling lines, pumps, staging tanks, fill lines, personnel gowning and behavior, and environmental quality all may contribute particulate matter to the filled product. Insufficient cleaning of product components and inappropriate selection of both container/closure systems and formula components may contribute to immediate and even long-term generation of particles. All of these contributors to particulate matter and final product quality may be evaluated by the methods in ⟨788⟩ or variations thereof.

The requirements of ⟨788⟩ should be considered for all parenteral preparations unless specifically exempted by chapter ⟨788⟩ or by monograph. What about dose forms for which ⟨788⟩ cannot be directly applied? Alternate methods and/or evaluation of process streams, vehicles, or components should be considered. For example, sterile suspensions with known active ingredient solids and light obscuration for the detection of extraneous matter are impossible to differentiate from product solids. For suspended solids above the nominal porosity of the ⟨788⟩ membranes, membrane microscopic assay will have significant interference from the solids. Only direct ⟨788⟩ evaluation of container/closure rinsates, and product vehicle is possible for these suspension systems by the ⟨788⟩ methods. One may explore alternate methodologies such as

1. particle determination of product treated with solvent to dissolve all product solids, or
2. use of centrifugation to separate product solids from the vehicle, or
3. use of selected screens or sieves to separate product solids from the product vehicle to minimize product solids interference.

Due diligence using ⟨788⟩ methods, variations of ⟨788⟩, and alternate method strategies for the investigation of best product design occurs during development and may be continued in selected evaluations during commercial production.

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Methodology for the determination of particulate matter in injections is contained in USP ⟨788⟩, which has been harmonized with the European Pharmacopoeia and the Japanese Pharmacopoeia. Sections on instrument standardization for light obscuration and other method details were excluded from the harmonized chapter. Chapter ⟨789⟩ *Particulate Matter in Ophthalmic Solutions* has not been harmonized. Chapter ⟨1788⟩ includes important instrument standardization and calibration information applicable to ⟨788⟩ and ⟨789⟩; it also includes recommendations for sample handling, laboratory environment, operator training, and general advice applicable to the microscopic method.

Chapter ⟨1⟩ requires injections to be essentially free from particulate matter that can be observed on visual inspection. The term "essentially free" has been difficult to define because particle detectability is influenced by their number and size, among other factors. The absolute limit of visibility, or detectability, is equivocal and depends upon the test conditions and the nature of the particulate matter. The lower end of the visible range certainly crosses over sub-visible detection capabilities in ⟨788⟩ and ⟨789⟩. Literature reports visibility extending to 50-µm, 100-µm, and 150-µm size (see *References 1* and *2*), and membrane assay can isolate and size particles to 1000 µm and larger.

Chapter ⟨788⟩ specifies limits for the sub-visible particulate matter content in injections in two size thresholds. Likewise, ⟨789⟩ establishes particle content expectations for ophthalmic solutions in two (Light Obscuration, or LO) or three (Membrane Microscope, or MM) size thresholds. The tests described in ⟨788⟩ and ⟨789⟩ are physical limit tests performed for the purpose of enumerating sub-visible particles (particulate matter) within specific size ranges (see *Figure 1*).

Size domains considered in USP <1>, <788>, <789>

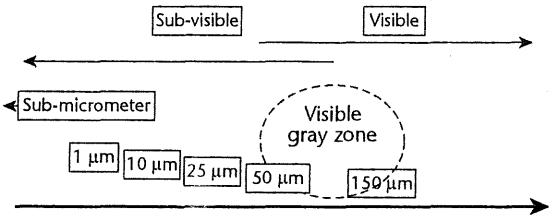

Figure 1. Increasing Probability of Visual Detection.

Chapter ⟨788⟩ states, "Particulate matter consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." Using the ⟨788⟩ test methods, any semi-solid to solid material (and even immiscible liquids) that trigger LO detector response above a selected size threshold will be tabulated.

There are two general categories of particulate matter sources: extrinsic and intrinsic. LO and MM methods will detect and tabulate particles of both categories. Extrinsic material is additive, foreign, and unchanging, and not part of the formulation, package, or assembly process.

Examples of extrinsic material include fibers, cellulosic matter, vegetative matter, corrosion products, paint/coatings, and building materials such as gypsum, concrete, metal, and plastic. Extrinsic particles are additive and generally non-changing over the life of the product, unless by fragmentation, swelling (hydration), or degradation. Fragments of rubber, plastic, metal, and glass are examples of extrinsic particulate matter deposited in the product during assembly or not removed in the container preparation process. However, if these typically extrinsic types have come from the specific package and/or process in a more consistent or chronic manner, then one may consider their presence to be an intrinsic variety, with a similar level of concern.

Intrinsic material is associated with the package, formulation ingredients, and process or assembly process. Intrinsic material may also be extraneous material carried by the package or process and insufficiently removed. Intrinsic material may indeed change upon aging, due to concentration change, degradation, and acceleration of reaction.

Intrinsic sources are inherent in the product and process—formulation, package, and commercial assembly steps. Intrinsic sources represent a variety of phenomena yielding unwanted substances, such as: (a) extraction, (b) leaching, (c) degradation of ingredient (active or excipient), (d) change of ingredient by precipitation/salt form/crystalline form, (e) change of package physical integrity, (f) change of impurity level, (g) change of micellar association, (h) oligamerization, and (i) package- and process-related materials not removed during product assembly. Combinations of all of the above and physical phenomena such as aggregation, sedimentation, and coalescence by matrix (oils, semi-solids) may bring smaller particles (<10 µm) into the detection zone of the test method (≥10 µm). Intrinsic sources of detectable particulate matter are of great concern, since the substance may be present, however, not evident until particles form over time, even long after lot release.

The intrinsic categorization should be recognized as different from inherent formulation character. Solution properties such as a slight haze or faint coloration of high concentration formulas and protein formulations are typical examples of an inherent characteristic of the product fluid, and while the condition may cause difficulties in inspection or LO assay, are not particle-related.

Certain solution formulations may not be easily analyzed by LO. The LO method may encounter problems with a product that does not have clarity and a viscosity approximating those of water. Further, formulation characteristics such as color, high viscosity or inherent formulation properties, such as shear-induced changes, may generate erroneous LO data. Similarly, products that produce air or gas bubbles when drawn into the LO sensor, such as bicarbonate-buffered formulations may generate erroneous data. For these product types the MM method may have to be used. Documentation demonstrating that the LO procedure is incapable of testing the test article or produces invalid results may aid regulatory filing strategy. It is expected that most test articles will meet the requirements on the basis of the LO test alone; however, it may be necessary to assay some test articles by the LO method followed by the MM method in order to reach a conclusion.

There may be a desire to test lower volumes of certain products, due to limited sample, high product cost, low container volume, or due to special fluid delivery characteristics. Examples include biopharmaceuticals, low-volume parenteral and ophthalmic products and formulations in novel packages intended for specific medical targets. The expectation is limits

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compliance for these products; however, one may employ methods validated by the manufacturer to demonstrate conformance with the test limits. Special low-volume "sippers" for LO sampling and the pooling of multiple containers may be necessary for these package presentations. Consider this example: a low-volume (100-µL) product is packaged in a pre-filled sterile syringe. The nature of the package allows simple delivery of the solution product and may be used for direct sampling, but the 100-µL volume precludes the pooling of the larger volumes (~25 mL) for the LO method. Direct sampling to a small membrane for microscopical counting and evaluating single and pooled package particle content may be the optimal means to collect data. Also in this example, careful statistical evaluation of the batch population using small sample volumes (but not doses) will be necessary to validate product acceptability.

LIGHT OBSCURATION PARTICLE COUNT TEST

Test Apparatus

The apparatus is a liquid-borne particle counting system that uses a light-obscuration sensor with a suitable sample-feeding device to deliver controlled aliquots of sample for analysis. Suspended particles in the sample fluid flowing between a light source and sensor produce changes in signal that are correlated to particle dimension. Due to the nature of the detection and counting system, air bubbles and immiscible liquids may block sufficient light to be recorded along with the target suspended particles. These artifacts must be diminished through proper preparation techniques. Solutions with excessive immiscible liquids may not be amenable to LO analysis. A variety of suitable devices of this type are commercially available. It is the responsibility of those performing the test to ensure that the operating parameters of the instrumentation are appropriate to the required accuracy and precision of the test result, the artifacts and interferences inherent in certain products and with certain methods of preparation are eliminated or accommodated. An example is a protein formulation that may form shear-induced semi-solids due to mixing and counted as "particles." Adequate training must be provided for those responsible for the technical performance of the test.

It is important to note that for Pharmacopeial applications the ultimate goal is that the particle counter reproducibly size and count particles present in the material under investigation. The instruments available range from systems where calibration and other components of standardization must be carried out by manual procedures to sophisticated systems incorporating hardware- and software-based functions for the standardization procedures. Thus, it is not possible to specify exact methods to be followed for standardization of the instrument, and it is necessary to emphasize the required end result of a standardization procedure rather than a specific method for obtaining this result. This section is intended to emphasize the criteria that must be met by a system rather than specific methods to be used in their determination. It is the responsibility of the user to apply the various methods of standardization applicable to a specific instrument. Critical operational criteria consist of the following.

SENSOR CONCENTRATION LIMITS

Use an instrument that has a concentration limit (the maximum number of particles per mL) identified by the manufacturer that is greater than the concentration of particles in the test specimen to be counted. The vendor-certified concentration limit for a sensor is specified as that count level at which coincidence counts due to the simultaneous presence of two or more particles in the sensor view volume compose less than 10% of the counts collected for 10-µm particles.

SENSOR DYNAMIC RANGE

The dynamic range of the instrument used (range of sizes of particles that can be accurately sized and counted) must include the smallest particle size to be enumerated in the products.

Instrument Standardization Tests

The following discussion of instrument standardization emphasizes performance criteria rather than specific methods for calibrating or standardizing a given instrument system. This approach is particularly evident in the description of calibration, where allowance must be made for manual methods as well as those based on firmware, software, or the use of electronic testing instruments. Appropriate instrument qualification is essential to performance of the test according to requirements. Since different brands of instruments may be used in the test, the user is responsible for ensuring that the counter used is operated according to the manufacturer's specific instruction; the principles to be followed to ensure that instruments operate within acceptable ranges are defined below. The following information for instrument standardization helps ensure that the sample volume accuracy, sample flow rate, particle size response curve, sensor resolution, and count accuracy are appropriate to performance of the test. Conduct these procedures at intervals of not more than six months.

SAMPLE VOLUME ACCURACY

Since the particle count from a sample aliquot varies directly with the volume of fluid sampled, it is important that the sampling accuracy is known to be within a certain range. For a sample volume determination, determine the dead (tare) volume in the sample feeder with particle-free water.[1] Transfer a volume of particle-free water that is greater than the sample volume to a container, and weigh. Using the sample feeding device, withdraw a volume that is appropriate for the specific sampler, and again weigh the container. Determine the sample volume by subtracting the tare volume from the combined sample plus tare volumes. Verify that the value obtained is within 5% of the appropriate sample volume for the test. Alternatively, the sample volume may be determined using a suitable Class A graduated cylinder (see *Volumetric Apparatus* ⟨31⟩). [NOTE—Instruments of

[1] Passed through a filter of 1.2-µm or finer nominal pore size.

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this type require a variable tare volume. This is the amount of sample withdrawn before counting. This volume may be determined for syringe-operated samplers by setting the sample volume to zero and initiating sampling, so that the only volume of solution drawn is the tare. Subtract the tare volume from the total volume of solution drawn in the sampling cycle to determine the sample volume.]

SAMPLE FLOW RATE

Verify that the flow rate is within the manufacturer's specifications for the sensor used. This may be accomplished by using a calibrated stopwatch to measure the time required for the instrument to withdraw and count a specific sample volume (i.e., the time between beginning and ending of the count cycle as denoted by instrument indicator lights or other means). Sensors may be operated accurately over a range of flow rates. Perform the *Test Procedure* below at the same flow rate as that selected for calibration of the instrument.

CALIBRATION

USP ⟨788⟩ specifies the use of dispersions of spherical particles of known sizes between 10 µm and 25 µm in particle-free water. More options follow:

Manual method: Calibrate the instrument with a minimum of three calibrators, such as near-mono-size polystyrene spheres having diameters of about 10, 15, and 25 µm, in an aqueous particle-free vehicle. The calibrator spheres must have a mean diameter of within 5% of the nominal diameters and be standardized against materials traceable to National Institute of Standards and Technology (NIST) standard reference materials.[2] The total number of spheres counted must be within the sensor's concentration limit. Prepare suspensions of the calibrator spheres in water at a concentration of 1000–5000 particles/mL, and determine the channel setting that corresponds to the highest count setting for the sphere distribution. This is determined by using the highest count threshold setting to split the distribution into two bins containing equal numbers of counts, with the instrument set in the differential count mode (moving window half-count method). Use only the central portion of the distribution in this calculation to avoid including asymmetrical portions of the peak. The portion of the distribution, which must be divided equally, is the count window. The window is bounded by threshold settings that will define a threshold voltage window of ±20% around the mean diameter of the test spheres. The window is intended to include all single spheres taking into account the standard deviation of the spheres and the sensor resolution, while excluding noise and aggregates of spheres. The value of 20% was chosen on the basis of the worst-case sensor resolution of 10% and the worst-case standard deviation of the spheres of 10%. Since the thresholds are proportional to the cross-sectional area of the spheres (and all particles) rather than the diameter, the lower and upper voltage settings are determined by the equations:

$$V_L = 0.64V_S$$

in which V_L is the lower voltage setting, and V_S is the voltage at the peak center, and

$$V_U = 1.44V_S$$

in which V_U is the upper voltage setting. Once the center peak thresholds are determined, use these thresholds for the standards to create a regression of log voltage versus log particle size, from which the instrument settings for the 10- and 25-µm sizes can be determined.

Automated method: The calibration (size response) curve may be determined for the instrument-sensor system by the use of validated software routines offered by instrument vendors; these may be included as part of the instrument software or used in conjunction with a microcomputer interfaced to the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a response curve equivalent to that attained by the manual method and if the automated calibration is validated as necessary by the user.

Electronic method: Using a multichannel peak height analyzer, determine the center channel of the particle counter pulse response for each standard suspension. This peak voltage setting becomes the threshold used for calculation of the voltage response curve for the instrument. The standard suspensions used for the calibration are run in order, and median pulse voltages for each are determined. These thresholds are then used to generate the size response curve manually or via software routines. The thresholds determined from the multichannel analyzer data are then transferred to the counter to complete the calibration.

SENSOR RESOLUTION

The particle size resolution of the instrumental particle counter is dependent upon the sensor used and may vary with individual sensors of the same model. Determine the resolution of the particle counter for 10-µm particles, using the 10-µm calibrator spheres. The relative standard deviation of the size distribution of the standard particles used is NMT 5%. Acceptable methods of determining particle size resolution are (1) manual determination of the amount of peak broadening due to instrument response; (2) using an electronic method of measuring and sorting particle sensor voltage output with a multichannel analyzer; and (3) automated methods.

Manual method: Adjust the particle counter to operate in the cumulative mode or total count mode. Refer to the calibration curve obtained earlier, and determine the threshold voltage for the 10-µm spheres. Adjust 3 channels of the counter to be used in the calibration procedure as follows:

Channel 1 is set for 90% of the threshold voltage.

Channel 2 is set for the threshold voltage.

Channel 3 is set for 110% of the threshold voltage.

[2] ASTM standard F658-00a provides useful discussions pertaining to calibration procedures applying near-monosize latex spheres.

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Draw a sample through the sensor, observing the count in *Channel 2*. When the particle count in that channel has reached approximately 1000, stop counting, and observe the counts in *Channels 1* and *3*. Check to see if the *Channel 1* count and *Channel 3* count are 1.68 ± 10% and 0.32 ± 10%, respectively, of the count in *Channel 2*. If not, adjust *Channel 1* and *Channel 3* thresholds to meet these criteria. When these criteria have been satisfied, draw a sample of suspension through the counter until the counts in *Channel 2* have reached approximately 10,000, or until an appropriate volume (e.g., 10 mL) of the sphere suspension has been counted. Verify that *Channel 1* and *Channel 3* counts are 1.68 ± 3% and 0.32 ± 3%, respectively, of the count in *Channel 2*. Record the particle size for the thresholds just determined for *Channels 1, 2,* and *3*. Subtract the particle size for *Channel 2* from the size for *Channel 3*. Subtract the particle size for *Channel 1* from the size for *Channel 2*. The values so determined are the observed standard deviations on the positive and negative side of the mean count for the 10-µm standard. One commonly used method for calculating the percentage of resolution of the sensor is the following:

$$\% \text{ resolution} = (100/D) \times [(S_{Obs})^2 - (S_{Std})^2]^{1/2}$$

in which S_{Obs} is the highest observed standard deviation determined for the sphere standard; S_{Std} is the supplier's reported standard deviation for the spheres; and D is the diameter, in µm, of the spheres as specified by the supplier. The resolution is NMT 10%.

Automated method: Software that allows for the automated determination of sensor resolution is available for some counters. This software may be included in the instrument or used in conjunction with a microcomputer interfaced to the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a resolution determination equivalent to the manual method and if the automated resolution determination is validated as necessary by the user.

Electronic method: Record the voltage output distribution of the particle sensor using a multichannel analyzer while sampling a suspension of the 10-µm particle size standard. To determine resolution, move the cursor of the multichannel analyzer up and down the electric potential scale from the median pulse voltage to identify a channel on each side of the 10-µm peak that has approximately 61% of the counts observed in the center channel. Use of the counter size response curve to convert the mV values of these two channels to particle sizes provides the particle size at within one standard deviation of the 10-µm standard. Use these values to calculate the resolution as described under *Manual Method*.

PARTICLE COUNTING ACCURACY—SYSTEM SUITABILITY

Determine the particle counting accuracy of the instrument, using *Method 1* [for sensors requiring the moving window half-count (MWHC) method for calibration], *Method 2* (for multichannel sensors), or *Method 3* for any instrument (manual comparison to membrane microscope method).

Method 1—MWHC instruments

Procedure: Prepare the suspension and blank using the USP Particle Count RS. With the instrument set to count in the cumulative (total) mode, collect counts at settings of ≥10 µm and ≥15 µm. Prepare the blank and suspension sample in the same manner. Degas the mixture by one of three means: by sonication (at 80–20 watts) for about 30 s, by allowing to stand, or by vacuum. Gently stir the contents by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis. Withdraw three consecutive volumes directly from the container. Historically, these have been volumes of NLT 5 mL each, due to instrument limitations and the desire to maximize sample volume. However, where desired, volumes may be utilized that meet the standardization criteria and address the sensitivities of the formulation. Obtain the particle counts, and discard the data from the first portion. [NOTE—Complete the procedure within 5 min.] Repeat the procedure, using the suspension in place of the blank. From the averages of the counts resulting from the analysis of the two portions of the suspension at ≥10 µm and from the analysis of the two portions of the blank at ≥10 µm, calculate the number of particles in each mL by the formula:

$$(P_S - P_B)/V$$

in which P_S is the average particle count obtained from the suspension; P_B is the average particle count obtained from the blank; and V is the average volume, in mL, of the 4 portions tested. Repeat the calculations, using the results obtained at the setting of NLT 15 µm.

Interpretation: The MWHC instrument meets the requirements for *Particle Counting Accuracy* if the count obtained at ≥10 µm and the ratio of the counts obtained at ≥10 µm to those obtained at ≥15 µm conform to the values that accompany the USP Particle Count RS. If the instrument does not meet the requirements for *Particle Counting Accuracy*, and adequate test volumes remain, repeat the procedure with them; if insufficient volumes remain, prepare new suspension and blank, and then repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test for *Particle Counting Accuracy*. If on the second attempt the system does not meet the requirements of the test, determine and correct the source of the failures, and retest the instrument.

Method 2—Multichannel instruments

Procedure: Use one of three standards: (1) a dilution of the USP Particle Count RS; (2) commercial preparation of standard calibrator spheres of nominal diameter 15–30 µm in a suspension containing between 50 and 200 particles/mL, certified by the manufacturer; or (3) a laboratory-prepared suspension of standard calibrator spheres having a nominal diameter of 15–30 µm, containing between 50 and 200 particles/mL. Use of non-USP standards 2 and 3 is acceptable when they are compliant with USP standardization criteria: five successive counts are NMT ±10% of stated concentration.

Degas the suspension by one of three means: by sonication (at 80–120 watts) for about 30 s, by allowing to stand, or by vacuum. Gently stir the contents by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis, and perform five counts on 5-mL volumes of the suspension, using the particle counter 10-µm size threshold. Obtain the mean cumulative particle count/mL.

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Interpretation: The instrument meets the requirements for *Particle Counting Accuracy* if the count obtained at ≥10 µm conforms to the values that accompany the USP Particle Count RS. If the instrument does not meet the requirements for *Particle Counting Accuracy*, repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test for *Particle Counting Accuracy*. If on the second attempt the system does not meet the requirements of the test, determine and correct the source of the failures, and retest the instrument.

Method 3—Alternate manual method

Procedure: Prepare a suspension of standard calibrator spheres having a nominal diameter of 15–30 µm, containing between 50 and 200 particles/mL. Degas the suspension by one of three means: by sonication (at 80–120 watts) for about 30 s, by allowing to stand, or by vacuum. Gently stir the contents by hand-swirling or by mechanical means taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis and perform five counts on 5-mL volumes of the suspension, using the particle counter 10-µm size threshold. Obtain the mean cumulative particle count per mL. Pipet a volume of this suspension containing 250–500 particles into a filter funnel prepared as described for *Microscope Particle Count Test, Filtration Apparatus*, below. After drying the membrane, count the total number of standard spheres collected on the membrane filter. This count should be within 20% of the mean instrumental count per mL for the suspension.

Test Environment

Specimens must be cleaned to the extent that the level of particles added by testing has a negligible effect on the outcome of the test.

Cleanse glassware, closures, and other required equipment, preferably by immersing and cleaning the items using warm, nonionic detergent solution. Rinse in flowing tap water, and then rinse again in flowing filtered water. Organic solvents may also be used to facilitate cleaning. [NOTE—These steps describe one way to clean equipment; alternatively, particulate-free equipment may be obtained from a suitable vendor.] Preferably, the test specimen, glassware, closures, and other required equipment are then finally rinsed with filtered water, using a hand-held pressure nozzle with final filter or other appropriate filtered water source within an environment protected by high-efficiency particulate air (HEPA) filters. While conducting the assay, non-shedding garments and powder-free gloves are worn within the HEPA environment. Perform the test in an environment that does not contribute any significant amount of particulate matter.

To collect blank counts, use a cleaned vessel of the type and volume representative of that to be used in the test. Place a 50-mL or more volume of filtered water in the vessel, and agitate the water sample in the cleaned glassware by inversion or swirling. [NOTE—A smaller volume, consistent with the article to be counted, can be used.] Degas by the same method to be used for the product samples, by one of three means; sonication (at 80–120 watts) for about 30 s, by vacuum, or by allowing to stand. Swirl the vessel containing the water sample by hand or agitate by mechanical means to suspend particles.

As described in ⟨788⟩: *Determine the particulate matter in 5 samples of filtered water, each of 5 mL. If the number of particles of 10-µm or greater size exceeds 25 for the combined 25 mL (NMT 1/mL), the precautions taken for the tests are not sufficient.*

It is recommended that when utilizing the test for the ⟨789⟩ method, the blank test should be considered failed, if in addition, the number of particles of 25 µm or greater in size exceeds 3.

Test Procedure

TEST PREPARATION

Prepare the test specimens in the following sequence. Outside of the unidirectional airflow cabinet to be used for the test, remove outer closures, sealing bands, but not the sealing closure. If shedding is noted to be an issue, remove or tape over the product labels as well. Place the samples in the test cabinet, and rinse the exteriors of the containers with filtered water as directed under *Test Environment*. Protect the containers from environmental contamination until analyzed. After proper mixing, open and withdraw, pour or otherwise sample the contents of the containers under test in a manner least likely to generate particles that could enter the test specimen. Contents of containers with removable stoppers may be poured out directly after removing the closures. Sampling devices having a needle to penetrate the unit closure may also be employed. Products packaged in flexible plastic containers may be sampled by cutting the medication or administration port tube or a corner from the unit with a suitably cleaned razor blade or scissors. Dry or lyophilized products may be constituted using their internal diluent, by removing the closure to add supplied product diluent or by injecting filtered water via hypodermic syringe. If test specimens are to be pooled, remove the closure and empty the contents into a clean container.

NUMBER OF TEST SPECIMENS

USP ⟨788⟩ provides the sampling plan according to product volume. For all products, regardless of volume, comprehensive experience regarding the integrity and consistency of the batch is gained throughout development allowing the proper sampling plans to be applied in commercial production that ensure sample selection is representative of batch quality. All batches must have sampling plans that accommodate desired statistical measures of batch quality and facilitate process control.

PRODUCT DETERMINATION

Depending upon the dosage form being tested, proceed as directed under the appropriate category below.

Liquid preparations

Volume in container less than 25 mL: Prepare the containers as directed under *Test Preparation*. Mix and suspend the particulate matter in each unit by inverting the unit 20 times. [NOTE—Because of the small volume of some products, it may be necessary to agitate the solution more vigorously to suspend the particles properly.] Open and combine the contents of 10 or more units in a cleaned container to obtain a volume of NLT 25 mL. Degas the pooled solution by one of three means: sonication for about 30 s, or by vacuum, or by allowing the solution to stand.

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Gently stir the contents of the container by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Remove four portions, that conform to the volumes utilized in the IST, and count the number of particles ≥10 µm and 25 µm. Disregard the result obtained for the first portion. [NOTE—For low-volume products, a pool of 15 or more units may be necessary to achieve a pool volume sufficient for four 5-mL sample aliquots. Smaller sample aliquots (i.e., <5 mL) can be used if the assay result obtained with the smaller aliquots is validated to give an assessment of batch suitability equivalent to that obtained with the 5-mL aliquots specified above.]

Volume in container 25 mL or more: Prepare the containers as directed under *Test Preparation*. Mix, and suspend the particulate matter in each unit by inverting the unit 20 times prior to opening the container for degassing. Degas the solution by one of three means: by sonication for about 30 s, or by vacuum, or by allowing the solution to stand. When sampling, ensure that the counter probe can be inserted into the middle of the solution. Gently stir the contents of the unit by hand-swirling or by mechanical means. Remove four portions, each of NLT 5 mL, and count the number of particles ≥10 µm and 25 µm. Disregard the result obtained for the first portion.

Dry or lyophilized preparations: Prepare the containers as directed under *Test Preparation*. Open each container, taking care not to contaminate the opening or cover. Constitute as directed by the labeling, according to the *Test Preparation*. Alternately, depending on the experiment, use:

- filtered water or
- an appropriate laboratory-filtered diluent if suitable.

Replace the closure, and manually agitate the container sufficiently to ensure dissolution of the drug. [NOTE—For some dry or lyophilized products, it may be necessary to let the containers stand for a suitable interval, and then agitate again to effect complete dissolution.] After the drug in the constituted sample is completely dissolved, degas the solution by sonication for about 30 s, or by exposing to vacuum, or by allowing the solution to stand. When sampling, ensure that the counter draw or sipping probe can be inserted into the middle of the solution. Gently stir the contents of the unit by hand-swirling or by mechanical means to mix and suspend any particulate matter. Proceed as directed for the appropriate unit volume under *Liquid preparations*, and analyze by withdrawing a minimum of four portions, each of NLT 5 mL, and count the number of particles ≥10 µm and 25 µm. Disregard the result obtained for the first portion.

Products packaged with dual compartments constructed to hold the drug product and a solvent in separate compartments: Prepare the units to be tested as directed under *Test Preparation* and according to product insert directions. Mix each unit as directed in the labeling, activating and agitating so as to ensure thorough mixing of the separate components and drug dissolution. Open and degas the units or pooled specimen to be tested by one of three means: sonication, or by vacuum, or by allowing the solution to stand. Proceed as directed for the appropriate unit volume under *Liquid preparations*, mix and suspend the particulate matter present in each unit by inversion or swirling or by mechanical means. Analyze by withdrawing a minimum of four portions, each of NLT 5 mL, and count the number of particles ≥10 µm and 25 µm. Disregard the result obtained for the first portion.

Products labeled "Pharmacy Bulk Package Not for Direct Infusion": Proceed as directed for *Liquid preparations* where the volume is 25 mL or more. Calculate the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average LO particle count/mL would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. [NOTE—For the calculations of test results, consider this maximum dose portion to be the equivalent of the contents of one full container.]

LO Calculations

Note that the particle limits must be reported as all particles ≥10 µm and all particles ≥25 µm. If the instrument has been configured to count in differential bins, such as ≥10–25 µm, ≥25–50 µm, ≥50 µm, etc., all bins ≥10 µm must be added to yield total ≥10-µm count; all bins ≥25 µm need to be added to yield total count ≥25 µm.

For example, the analyst has counted the test samples in eight bins: a) ≥10–15 µm, b) ≥15 µm–25 µm, c) ≥25µm–40 µm, d) ≥40 µm–75 µm, e) ≥75 µm–100 µm and f) ≥100 µm. They would then calculate $P_{\Sigma\geq 10}$ as:

$$P_{\Sigma\geq 10} = P_{\geq 10\text{–}15\ \mu m} + P_{\geq 15\text{–}25\ \mu m} + P_{\geq 25\text{–}40\ \mu m} + P_{\geq 40\text{–}75\ \mu m} + P_{\geq 75\text{–}100\ \mu m} + P_{\geq 100\ \mu m}$$

POOLED SAMPLES

Average the counts from the two or more aliquot portions analyzed. Calculate the number of particles in each container by the formulas:

$$P_{\Sigma 10} V_T / V_A n$$

$$P_{\Sigma 25} V_T / V_A n$$

in which $P_{\Sigma 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma 25}$ is the average particle count per threshold obtained from all portions ≥25 µm analyzed. V_T is the volume of pooled sample, in mL; V_A is the volume, in mL, of each portion analyzed; and n is the number of containers pooled.

INDIVIDUAL SAMPLES

Average the counts obtained for the 5-mL or greater aliquot portions from each separate unit analyzed, and calculate the number of particles in each container by the formulas:

$$P_{\Sigma 10} V / V_A$$

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$$P_{\Sigma 25}V/V_A$$

in which $P_{\Sigma 10}$ is the average particle count per threshold obtained from all portions analyzed; and $P_{\Sigma 25}$ is the average particle count per threshold obtained from all portions ≥25 µm analyzed. V is the volume, in mL, of the tested unit; and V_A is the volume, in mL, of each portion analyzed.

INDIVIDUAL UNIT SAMPLES

Average the counts obtained for the two or more 5-mL aliquot portions taken from the solution unit. Calculate the number of particles in each mL of product solution taken by the formulas:

$$P_{\Sigma 10}/V$$

$$P_{\Sigma 25}/V$$

in which $P_{\Sigma 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma 25}$ is the average particle count per threshold obtained from all portions ≥25 µm analyzed. V is the volume, in mL, of the portion taken.

For all types of product, if the tested material has been diluted to decrease the viscosity, the dilution factor must be accounted for in the calculation of the final test result. For all test results, the particle count ≥10 µm represents all threshold bin counts.

MEMBRANE MICROSCOPE PARTICLE COUNT TEST

The microscope particulate matter test may be applied to both large-volume and small-volume parenteral injections and to ophthalmic solution products as well. This test enumerates essentially solid[3] particulate matter ≥10 µm in these products, after collection, rinsing and drying on a micro-porous membrane filter. Since a wide range of test aliquots may be utilized, particle counts may be determined on a per-volume or per-container basis without dilution or extrapolation.

In the performance of the membrane microscope assay, one estimates the size of retained solids viewed at 100× magnification, tabulating them into specific size categories. In this process, one may encounter materials on the membrane surface that do not appear solid or substantial, showing little or no surface relief such as a "stain" or discontinuity on the membrane. Chapter ⟨788⟩ advises not to attempt to size or enumerate such semi-solid particles, due to historical comment from LVP terminal sterilization manufacturers that encountered stain-like brown residues after heat sterilization of Dextrose solutions. However, if not sampling a carbohydrate solution or similarly-performing formulation, recognizing the presence of such material adds a measure of formulation robustness. Consistent evidence of such materials may be indication that further development research is warranted to understand their content. The nature of these materials and subsequent decision to count or investigate must be based upon product formulation experience. Interpretation of microscopical enumeration may be aided by testing a sample of the solution by the LO particle count or a validated, alternate method.

The *Test Apparatus* is described in ⟨788⟩. Additional comments are:

- Use a compound binocular microscope that corrects for changes in interpupillary distance by maintaining a constant tube length.
- The objective must be of 10× nominal magnification, a planar achromat or better in quality, with a minimum 0.25 numerical aperture.
- The objective must be compatible with an episcopic illuminator attachment.
- The eyepieces must be matched. In addition, one eyepiece must be designed to accept and focus an eyepiece graticule. The microscope must have a mechanical stage capable of holding and traversing the entire filtration area of a 25-mm or 47-mm membrane filter.
- Two illuminators are required. Both illuminators must be of sufficient output to provide a bright and even source of illumination and may be equipped with blue daylight filters to decrease operator fatigue during use.
- The USP graticule as described in ⟨788⟩ is used.

Stage Micrometer

Graduated in 10-µm increments, utilized each day-of-use. For initial calibration, utilize a stage micrometer that is certified by NIST to verify the USP graticule installation. Thereafter, for daily calibration/verification, one may utilize a commercial stage micrometer graduated in 10-µm increments to verify proper setup.

Filtration Apparatus

Use a filter funnel suitable for the volume to be tested, generally having an inner diameter of about 16 mm for 25-mm membranes or about 37 mm for 47-mm membranes. The funnel is made of plastic, glass, or stainless steel. Use a filter support made of stainless steel screen or sintered glass as the filtration diffuser. A solvent dispenser capable of delivering solvents filtered through a membrane filter at a range of pressures from 10 psi to 80 psi.

[3] Soft particles and semi-solid substances may also be retained.

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Membranes

As described by ⟨788⟩; however, finer pore size selections will have smoother surfaces, facilitating the microscopical examination; however, may impede more viscous sample fluid during the assay.

Test Environment

Following discussion is recommended operational detail to enhance the conductance of the MM assay.

It is ideal to use two unidirectional airflow hood (UAFH) or other unidirectional airflow enclosures, one for "wet" sample preparations, and the other an enclosure for the microscope counting phase. The UAFH having a capacity sufficient to envelop the area in which the analysis is prepared. The UAFH provides HEPA-filtered air which typically contains NMT 100 particles (0.5 µm or larger) per cubic foot. A blank determination is necessary at the beginning of each test sequence to verify minimal contribution from the background, equipment and personnel operations. What is the definition of a test sequence? Should it be one per shift, one per product family, one per series of filtrations (manifold) or one per sample? Any of these definitions may be suitable, dependent upon the operational needs of the lab system. The ability to clean glassware between samples, the number of different products being run, and the volume of samples through the lab will determine the appropriate control. However, consider the blank to be a system suitability check, and if it fails, all samples run prior to it up to the previous blank, are suspect.

To determine the blank count, duplicate the sample preparation process, in regard to the apparatus and membrane types. Assemble a clean filtration apparatus with a fresh membrane, rinse the interior with filtered water to drain, then deliver a 50-mL or more volume of filtered water to the filtration funnel while applying vacuum, and draw the entire volume of water through the membrane filter. Remove the membrane from the filter funnel base, and place onto a holding device as will be used for test specimens; typically atop a strip of double-sided tape on a microscope slide or in a commercial membrane holder or Petri dish. After allowing the membrane to dry (it must be counted dry), examine the entire filtration area microscopically at a magnification of 100×. If NMT 20 particles ≥10 µm and NMT 5 particles ≥25 µm or larger are present within the filtration area, the background particle level is sufficiently low for performance of the microscope assay for ⟨788⟩. If particle load exceeds these limits, repeat the procedure.

There is value in further limiting the background for both ⟨788⟩ and ⟨789⟩ testing in regard to good laboratory practice, and more specifically in regard to the ⟨789⟩ ≥25 µm and ≥50 µm limits, which may be considered more restrictive than injectable limits in consideration of total particle content allowed for the (usually) small unit volumes. Compare total particle load for a small SVI, small LVI versus a 5-mL ophthalmic product in *Table 1*.

Table 1. Comparison of Total At-Limit Load for Selected Products

Size Limit	Blank Count	SVI, 5 mL	LVI, 125 mL	Ophthalmic Product, 5 mL
≥10 µm	20	3000 particles	1500 particles	250 particles
≥25 µm	5	300 particles	250 particles	25 particles
≥50 µm	Not defined	N/A	N/A	10 particles

Therefore, in smaller volume ⟨789⟩ applications and in low-particle count injectable product, the laboratory should strive for consistent and lower blank counts such as NMT 5 ≥10 µm, NMT 1 ≥25 µm and none ≥50 µm per blank.

Throughout the operational procedure (in the HEPA environment), it is preferable to use powder-free gloves, and low-shedding clothing. Prior to conducting the test, clean the work surfaces of the unidirectional flow enclosure with an appropriate filtered solvent. Glassware and equipment should be rinsed successively with a warm, residue-free solution of detergent, hot water, filtered distilled or deionized water, and isopropyl alcohol. [NOTE—Prior to use, pass the distilled or deionized water and the isopropyl alcohol through membrane filters 0.2-µm or finer nominal pore size.] Perform the rinsing in the unidirectional airflow enclosure. Allow the glassware and filtration apparatus to dry in the unidirectional airflow enclosure, upstream of all other operations. Preferably, the enclosure is located in a separate room that is supplied with filtered air-conditioned air and maintained under positive pressure with respect to the surrounding areas.

MICROSCOPE PREPARATION

The microscope optical alignment and illumination are critical for success of this method. Although it is not difficult to differentiate a 10-µm from a 25-µm particle at 100× with reflected light, the decision regarding the boundary at each size category will be difficult with poor equipment, maintenance or optical alignment. Also operator fatigue is caused by poor microscope alignment. We will have to make decisions such as "is this particle 9 µm or 11 µm?" and "is that particle 24 µm or 26 µm?" Optimized system resolution, that is, the ability to discern discrete points of minimal separation, relies upon good optical systems, aligned well. Factors including instrument cleanliness, resolution, e.g., objective N.A.,[4] focus of both eyepieces and the graticule will play significant roles in attaining best images. In consideration of optimizing the use of the binocular compound microscope, it is best to utilize operators familiar with the instrument, and comfortable with alignment. The operator conducting the method should align the optics and illumination for their use, with supervisory/trainer approval.

[4] N.A. is numerical aperture, an indicator of optical light-gathering capability, and thus resolution. High N.A. correlates to high resolution.

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It is recommended to start with alignment of the microscope for a typical transmitted illumination observation using a known sample. Any specimen familiar to the operator will suffice; however, a common particle count reference standard suspension such as the USP Particle Count RS is a recommended selection, since it is also utilized in method system suitability evaluation. A drop of the USP Particle Count RS is placed between a glass microscope slide and cover slip and viewed microscopically.[5] With appropriate interpupillary distance and a comfortable sitting position at the microscope, the operator examines the fields of suspended spheres. One should observe the small standard spheres crisply in a combined field (with ease) for both eyes. One attains crisp focus and ease of view after separate focal adjustment of each eyepiece focus for a single point on the specimen.

Rotate the graticule in the right microscope eyepiece so that the linear scale is located at the bottom of the field of view, bringing the graticule into sharp focus by adjusting the right eyepiece diopter ring while viewing an out-of-focus specimen. Focus the microscope on a specimen, looking through the right eyepiece only. Then, looking through the left eyepiece, adjust the left eyepiece diopter to bring the specimen into sharp focus.

When the operator is not comfortable using the microscope or does not attain an equivalent crisp focus for each eye in a merged field of view, the counting will become a difficult experience and fatigue and flawed size comparison will result.

Nothing is better for preparing the operator for counting particles than to examine a test membrane as a positive control. Seasoned microscopists may not require this step, but for new operators or individuals conducting many different types of methods in the modern laboratory, familiarization is a prudent exercise. A filter membrane of the type being used for the method, such as a 25-mm color-contrast, plain 0.45-µm nominal pore size, containing particles, is a good choice. This may be a sample from previous method that contains a variety of particle types, or one prepared for familiarization. This positive test control will contain natural particles (flakes, threads, equant particles, various colors/opacity, a range of sizes, etc.) to effectively refresh the operator's sensitivity and facilitate microscope and illumination alignment for optimal viewing.

One would examine the membrane preparation, locate a typical array of particles and first bring the illumination into good alignment:

1. Adjust the external, incident illumination at an oblique angle (10°–20° to the method) so that an even ellipse of reflected light is visible on the membrane and an even illumination evident through the eyepiece field of view (even across the full field). Shadows will be evident from larger particles, such as those with z axis >5µm (z axis is the microscope optical axis).
2. Adjust the internal episcopic brightfield illuminator to yield an even illumination at a high setting on the transformer control, but more importantly, when dialing down the illumination one observes the evident shadow from the larger particles. In this manner, the high reflectivity of flat, glassy particles (find one) and the distinct shadows of more equant (x:y:z ~ 1:1:1) particles is evident.

USING THE CIRCULAR DIAMETER GRATICULE

The USP graticule is specifically fabricated for each microscope. The relative error of the graticule used must be ±2% and is initially measured with an NIST-certified stage micrometer. To accomplish this, align the graticule micrometer scale with the stage micrometer so that they are parallel. (Compare the scales, using as large a number of graduations on each as possible.) Read the number of graticule scale divisions, *GSD*, compared to stage micrometer divisions, *SMD*. Calculate the relative error by the formula:

$$100[(GSD - SMD)/SMD]$$

A relative error of ±2% is acceptable and verifies good alignment, focus and proper magnification. Thereafter, a day-of-use verification by the microscope operator with the NIST stage micrometer or commercial stage micrometer is sufficient to demonstrate proper setup.

The basic technique of measurement applied with the use of the circular diameter graticule is to count all particles 10 µm and larger, further categorizing in ≥10 µm and ≥25 µm. The circular zone or graticule field of view is a useful zone for active sizing and counting. Particles are compared to the linear scale and/or circles to determine their size in equivalent circular diameter. This is conducted by transforming mentally the image of each particle into a circle and then comparing to the 10- and 25-µm graticule reference circles. The sizing process is carried out without superimposing the particle on the reference circles; particles are not moved from their locations within the graticule field of view (the large circle) for comparison to the reference circles. Compare the area of the particle being sized to that of the black or transparent circles. Use the area of the clear graticule reference circles to size white or transparent particles. Use the area of the black reference circles to size dark particles. The intent of comparing particles to an equivalent circular diameter is correlation to the LO particle sizing methodology, for which many manufacturers have extensive databases. In practice, particles with nearly circular areas will correlate well with the graticule circle diameters. For particles with one long axis, such as rods and needles, the conversion to circular area will produce more significant bias to smaller estimated sizes. It may be simpler, and most conservative, to count particles in longest chord. To use an extreme example, the total count of mono-dispersions of fine needle crystals would vary greatly dependent upon the size determination utilized.

In order to properly focus the ocular lenses and attain balanced single-field view, each operator must bring the USP graticule lines into sharp focus by adjusting the eyepiece diopter ring (it helps to have an "infinite" view, or out-of-focus specimen). Next, focus the microscope on a specimen, through this same eyepiece, and then looking only through the other eyepiece, adjust its diopter ring to bring the specimen into sharp focus. The USP graticule and specimen particles are now in focus on a well-balanced illumination field.

Preparation of Filtration Apparatus and *Test Preparations* are covered by ⟨788⟩. Further, prepare the test specimens in the following sequence. Outside of the unidirectional airflow cabinet to be used for the test, remove outer closures, sealing bands, and remove or tape over labels. Rinse the exteriors of the containers with filtered water as directed under *Test Environment.* Protect the containers from environmental contamination until analyzed.

[5] The microscope objective requires a defined cover slip thickness, nominally 170 µm, or No. 1 ½.

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Within the HEPA cabinet, open and withdraw, pour, or otherwise sample the contents of the containers under test in a manner least likely to generate particles that could enter the test specimen. Contents of containers with removable stoppers may be withdrawn directly by removing the closures. Sampling devices having a needle to penetrate the unit closure may also be employed. Products packaged in flexible plastic containers may be sampled by cutting the medication or administration port tube or a corner from the unit with a suitably cleaned razor blade or scissors.

For all products, regardless of volume, comprehensive experience regarding the integrity and consistency of the batch is gained throughout development, allowing the proper sampling plans to be applied in commercial production that ensure sample selection is representative of batch quality. All batches must have sampling plans that accommodate desired statistical measures of batch quality and facilitate process control.

PRODUCT PARTICLE COUNT DETERMINATION

Depending upon the dosage form being tested, proceed as directed under the appropriate category below.

Liquid preparations: Thoroughly mix the units to be tested by inverting 20 times. Open the units in a manner consistent with the generation of the lowest possible numbers of background particles. For products less than 25 mL in volume, one may open them and drain to the filtration barrel individually, or combine the contents of 10 or more units in a cleaned container. [NOTE—When pooling containers, these must be included in the blank determination step.] Filter large-volume injection units individually. Small-volume injection units having a volume of 25 mL or more may be filtered individually.

Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If the volume of solution to be filtered exceeds the volume of the filtration funnel, add, stepwise, a portion of the solution until the entire volume is filtered. It is prudent to maintain the liquid volume in the filtration funnel above one-half of the funnel volume between refills, especially if the partial count procedure is to be used (see *Enumeration of Particles, Partial Count Procedure*, below). [NOTE—This is necessary in order to ensure even distribution of particles on the analytical membrane.] After the last addition of solution, begin rinsing the walls of the funnel by directing a low-pressure stream of filtered water in a circular pattern along the walls of the funnel, and stop rinsing the funnel before the volume falls below about one-fourth of the fill level. Maintain the vacuum until all the liquid in the funnel is gone.

Remove the filtration funnel from the filtration base while maintaining vacuum, then turn the vacuum off, and remove the filter membrane with non-serrated forceps. Place the filter in the prepared holder and label with sample identification. Allow the filter to air-dry in the unidirectional airflow enclosure with the cover ajar.

Dry or lyophilized preparations: Prepare the containers as directed under *Test Preparation.* Open each container taking care not to contaminate the opening or cover. Constitute as directed by the labeling, according to the *Test Preparation*. Alternatively, depending on the experiment, use:

- filtered water or an appropriate laboratory-filtered diluent if suitable.

Products packaged with dual compartments constructed to hold the drug product and a solvent in separate compartments: Activate each unit as directed in the labeling, agitating the contents sufficiently to ensure thorough mixing of the separate components, and then proceed as directed for *Liquid Preparations*.

Pharmacy bulk packages or multiple-dose containers: For *Products Labeled "Pharmacy Bulk Package—Not for Direct Infusion"* or for multiple-dose containers, proceed as directed for *Liquid preparations*, filtering the total unit volume.

Calculate the test result on a portion that is equal to the maximum dose given in the labeling. Consider this portion to be the equivalent of the contents of one full container. For example, if the total bulk package volume is 100 mL and the maximum dose listed is 10 mL, the microscope total unit volume count test result would be multiplied by 0.1 to obtain the test result for the 10-mL dose volume. [NOTE—For calculation of the test result, consider this portion to be the equivalent of the contents of one full container.]

Enumeration of Particles

The microscope test described in this section is flexible in that typical artifacts such as air and immiscible liquids do not interfere with the final count. The method has a broad size detection and counting range, if applying the partial count procedure. This method may be used where all particles on an analysis membrane surface are counted or where only those particles on some fractional area of a membrane surface are counted.

TOTAL COUNT PROCEDURE

The microscope method can be tedious (boring), imprecise (poor agreement within and between labs), and particle sizing can be inaccurate for non-spherical or equant particle shapes. Operator fatigue is promoted by poor ergonomic fit (chair height), by poor or imbalanced ocular focus and by excessive eye movement. Restricting the eye movement to a field-defining graticule such as the USP counting graticule restricts eye movement to the central one-third field of view. This significantly limits eye movement and thus fatigue.

Sample size is an important consideration in counting precision. Care must be taken to sample many containers within a batch for good representation of the particle distribution. Accordingly, the portion of the individual package sampled is key. Particles may float or settle. Sampling only the first 25 mL of an LVP or sampling without adequate and recent mixing is a mistake, and will lead to serious under-counting. Sampling whole, well-mixed containers with the particles in suspension is the best approach.

Counting the isolated particles is an important parameter. Counting all of the particles retained on the membrane is certainly the best approach, and then the simple problem is determining the correct size for placement into the threshold bins, 10 µm and 25 µm. This will be increasingly important for methods utilizing additional bins for population determination, such as 5 µm, 50 µm, 100 µm, etc. Note that the particle limits for ⟨788⟩ and ⟨789⟩ must be reported as all particles ≥10 µm and all particles ≥25 µm. If the lab method has been configured to count in several bins, such as ≥10–25 µm, ≥25–50 µm, ≥50 µm,

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etc., all bins ≥10 µm must be added to yield total ≥10-µm count; all bins ≥25 µm need to be added to yield total count ≥25 µm. Using a number of narrow size bins may be beneficial in product improvement efforts to separate particle groups.

In performance of a total count, the graticule field of view (GFOV) is defined by the large circle of the graticule, and the vertical crosshair is used as a counting target. Scan the membrane in paths that cover the effective filtration area (EFA), adjoining but not overlapping previous scan paths. Repeat this procedure, tabulating particle counts minimally in the ≥10 µm–25 µm and ≥25-µm thresholds, moving across the membrane until all particles on the membrane within the EFA are counted. Record the total number of particles that are ≥10 µm–25 µm and the number that are ≥25 µm or larger.

For large-volume products, calculate the particle count, in particles per mL, for each unit tested by the formulas:

$$P_{\Sigma\geq 10}/V$$

$$P_{\Sigma\geq 25}/V$$

in which $P_{\Sigma\geq 10}$ is the total particle count obtained from all portions analyzed, $P_{\Sigma\geq 25}$ is the total particle count obtained from all portions ≥25 µm analyzed, and V is the volume, in mL, of the solution tested.

For example, the analyst has counted the test samples in four bins: (a) ≥10–25 µm, (b) ≥25 µm–50 µm, (c) ≥50 µm –100 µm, and (d) ≥100 µm. They would then calculate as:

$$P_{\Sigma\geq 10} = P_{\geq 10\text{–}25\ \mu m} + P_{\geq 25\ \mu m\text{–}50\ \mu m} + P_{\geq 50\ \mu m\text{–}100\ \mu m} + P_{\geq 100\ \mu m}$$

For small-volume products, calculate the particle count, in particles per container, by the formulas:

$$P_{\Sigma\geq 10}/n$$

$$P_{\Sigma\geq 25}/n$$

in which $P_{\Sigma\geq 10}$ is the total particle count obtained from all portions analyzed, $P_{\Sigma\geq 25}$ is the total particle count obtained from all portions ≥25 µm analyzed, and n is the number of units pooled (1 in the case of an individual unit).

PARTIAL COUNT PROCEDURE

When encountering a membrane full of particles, the task of properly counting all of them is daunting. Consider that an SVP with an at-limit content of small particles, sampled in a 10-vial pool would have 30,000 10-µm particles on the membrane. Partial or statistical counting of the membrane effective filtration area may be the only means to attain reasonable results. Partial counting should not be used to reduce count times, just as a means to estimate the total load on a high-count isolate. A field-defining device, such as grids on the membrane surface or an ocular graticule field of view have been used reliably. An ocular graticule provides a sharp boundary for area definition. Gridded membrane lines are rather broad and have ink-spatter that may be taken for particulate matter.

Which portions and how much of the EFA should be counted? In consideration of 25-mm membranes, the EFA is 16-mm diameter using typical commercial filtration funnels, and thus (pi × r^2) = 201 mm². Based upon earlier proposals from the HIMA committee and discussion by Draftz (see *Reference 3*), acceptable confidence intervals (Poisson distribution, 2 standard deviations) dictate that for samples with less than 1000 particles, the imprecision of statistical counting is objectionable. Full count is recommended for such samples. For samples with more than 1000 particles on the isolate membrane, using a 25-mm membrane, a reasonable estimate of particle population is attained using 20 GFOV. If a smaller confidence interval about the result is desired a larger number of fields and particles may be counted.

For 47-mm membranes the EFA is 37 mm. These larger diameter membranes may be selected for formulations needing more membrane surface area (having slow flow characteristics through 25-mm membranes) the EFA = (pi × r^2) = (pi × 18.5 mm²) = 1075 mm². Thus, for 47-mm membrane EFAs, many more GFOVs must be counted to attain similar confidence. Using 100 GFOVs for partial counting of 47-mm membranes provides similar statistical confidence to the 20-GFOV/25-mm approach. Accordingly, when a particle load of 1000 or less is present, a full count is recommended.

When a partial count of particles on a membrane is to be performed the analyst must first ensure that an even distribution of particles is present on the membrane. This is assessed by rapid scanning at 50× to qualitatively scan for heterogeneity or clumps of particles. If heterogeneity is observed, one should perform a full count on the membrane. Next, count the 10-µm or larger particles in one GFOV at the edge of the filtration area as well as one in the center of the membrane. The number of ≥10-µm or larger particles in the GFOV with the highest total particle count must not be more than twice that of the GFOV with the lowest particle count. Fully count the membrane failing these criteria.

To perform a partial count of the particles on a membrane, include all particles ≥10–25 µm and ≥25 µm within the GFOV and those that are in contact with the right side of the GFOV circle. Do not count particles outside of the GFOV. Ignore those that touch the left side of the GFOV circle. The dividing line between right and left sides of the GFOV circle is the vertical cross hair and is a useful counting line. [NOTE—Make the best possible judgment on particle size without changing the membrane position, microscope magnification or illumination.]

Start at the center edge of the filtration area and begin counting adjacent GFOVs. When the other edge of the filtration area is reached, move one GFOV toward the top of the filter and continue counting GFOVs by moving in the opposite direction. Moving from one GFOV to the next can be accomplished by one of two methods. One method is to define a landmark (particle or surface irregularity in the filter) and move over one GFOV in relation to the landmark. A second method is to use the vernier on the microscope method to move 1 mm between GFOVs. To facilitate the latter, adjust the microscope x- and y-method positioning controls to a whole number at the starting position at the center right edge of the filtration area, then each GFOV will be one whole division of movement of the x-method positioning control. If the top of the filtration area is reached before the desired number of GFOVs is reached, begin again at the right center edge of the filtration area one GFOV lower than the

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first time. This time move downward on the membrane when the end of a row of GFOVs is reached. Continue as before until the number of GFOVs is complete.

For large-volume products, extrapolate the total count of particles per mL by the formulas:

$$P_{\Sigma 10}A_T/A_PV$$

$$P_{\Sigma 25}A_T/A_PV$$

in which $P_{\Sigma 10}$ is the total particle count obtained from all fields of view and all size thresholds; $P_{\Sigma 25}$ is the total particle count obtained from all fields of view and all size thresholds ≥25 µm; A_T is the filtration area, in mm², of the membrane (inner filtration barrel diameter); A_P is the partial area counted, in mm², based on the number of graticule fields counted (GFOV area × number of GFOV counted); and V is the volume, in mL, of solution filtered.

For a solution pool (for small-volume product units containing less than 25 mL) or for a single unit of a small-volume product, extrapolate the total count of particles per unit by the formulas:

$$P_{\Sigma 10}A_T/A_Pn$$

$$P_{\Sigma 25}A_T/A_Pn$$

in which $P_{\Sigma 10}$ is the total particle count obtained from all fields of view and all size thresholds, $P_{\Sigma 25}$ is the total particle count obtained from all fields of view and all size thresholds ≥25 µm, and n is the number of units counted (1 in the case of an individual unit). For all types of product, if the tested material has been diluted to decrease viscosity, the dilution factor must be accounted for in the calculation of the final test result.

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⟨1790⟩ VISUAL INSPECTION OF INJECTIONS

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1. SCOPE

1.1 Introduction

This chapter provides guidance on the inspection of injections for visible particles. The terms particle, particulates, and particulate matter are equivalent and do not have different meanings when used in this chapter. Particulate matter is defined in *Particulate Matter in Injections* ⟨788⟩ as "mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." Visual inspection is a probabilistic process and the specific detection probability observed for a given product for visible particles will vary with differences in product formulation, particle characteristics, and package design. The methods discussed in this chapter are also applicable to the detection of other visible defects not the subject of *Visible Particulates in Injections* ⟨790⟩, but critical to a qualified, comprehensive inspection process. These include, but are not limited to, container integrity defects such as cracks, misplaced stoppers, or incomplete seals, any of which may compromise the sterility of the product. Additional container defects (*1*), as well as other product characteristics such as fill level, discoloration, or clarity may also be detected during visual inspection, and nonconforming units should be rejected using the methods described in this chapter. Inspection for these other quality attributes often occurs at the same time as the inspection for particles. The primary focus of this chapter is a manual reference inspection method; however, semi-automated and automated methods are also discussed and permitted by the pharmacopeia.

1.2 Related Chapters

Injections and Implanted Drug Products ⟨1⟩ provides an overview of injectable dosage forms and the quality tests associated with them. Another chapter, ⟨790⟩, has been added to the *USP–NF* to provide a clear definition of routine inspection procedures for injectable products; the goal is to comply with the expectation that products be essentially free of visible particulate matter. Additionally, information on the detection of subvisible particulates is provided in *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, ⟨788⟩, and *Particulate Matter in Ophthalmic Solutions* ⟨789⟩. *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩ and *Methods for the Determination of Particulate Matter in Injections and Ophthalmic Solutions* ⟨1788⟩ provide additional supporting information on measurement methods for subvisible particles.

1.3 Defect Prevention

Although this chapter focuses on detection and removal of product units that show evidence of visible particles, the need for preventing such contamination should not be overlooked. No inspection process, manual or automated, can guarantee complete removal of all visible particulate matter or other visible defects; thus, prevention of such defects is an important consideration. Good process and product design, along with environmental control, are necessary to ensure the reliable production of products with a low particle burden. To ensure the control of defects throughout the process, manufacturers should consider an inspection life-cycle approach (*2*). This approach begins with developing quality attributes based on incoming component specifications, followed by component-level acceptance testing. It extends to component preparation and product-filling procedures, followed by 100% in-process inspection of filled product, and concluding with final acceptance sampling and testing of the finished product. The life-cycle approach must extend to purchased, ready-to-use components such as containers or closures, where there is no opportunity for subsequent particle removal after receipt and before filling to secondary packaging (e.g., trays, tubs, covers, and bags). Stability and retention sample inspection, customer complaint evaluation, and in-house investigative procedures support this integrated approach. The inspection life-cycle is composed of, and supported by, sub-cycles involving qualification, maintenance, personnel training, categorization of the fill particles for subsequent defect characterization (and possible identification) by forensic analytical methods, and the use of standards and defect samples within each of the critical areas. The final element of the life-cycle is a feedback loop of trending and data review from each of these process areas, resulting in a mechanism that supports continuous process improvement.

2. BACKGROUND

2.1 Inspection Process Capability

Visual inspection of injections is necessary to minimize the introduction of unintended particles to patients during the delivery of injectable medications. Such inspection also offers the opportunity to reject containers whose integrity has been

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compromised, such as those with cracks or incomplete seals, which pose a risk to the sterility of the product. The desire to detect these defects, despite their very low frequency and the randomness of their occurrence, has resulted in the longstanding expectation that each finished unit will be inspected (100% inspection). Although zero defects is the goal and this should drive continuous process improvement, zero defects is not a feasible specification for visible particles given current packaging components, processing capability, and the probabilistic nature of the inspection process.

The detection process is probabilistic: the likelihood of detection is a cumulative function of visible attributes such as particle quantity, size, shape, color, density, and reflectivity. Understanding human performance is therefore critical to establishing visual inspection criteria. Individual receptors in the eye have a theoretical resolution of 11 µm, but typical resolving power is reported as 85–100 µm (*3*). Analysis of inspection results pooled from several studies (*4–6*) conducted with standards prepared with single spherical particles show that the probability of detection (PoD) for a seeded sample with a single 50-µm particle in a clear solution contained in a clear 10-mL vial utilizing diffuse illumination between 2,000 and 3,000 lux is only slightly greater than 0%. The detection probability increases to approximately 40% for a seeded standard with a 100-µm particle and the threshold for routine, reliable detection (≥70% PoD) of individual visible particles is often near 150 µm in diameter (*4*) and typically exceeds 95% for particles that are 200 µm and larger. The PoD for fibers of similar length is less than that discussed for spherical particles above, with reliable detection often commencing at or above 500 µm. Thus, in a qualified visual inspection system, the vast majority of nonfibrous particles that might go undetected and be introduced into the pharmaceutical supply chain will be smaller than 200 µm. Changes to the container (e.g., increasing size and opacity), formulation (e.g., color and clarity), fill level, and particle characteristics beyond size (e.g., color, shape, and density) will all affect the PoD that can be achieved for a specific product and package (*6*).

2.2 Patient Risk

A complete review of the medical literature is beyond the scope of this chapter, but the effect of particles on the patient must be considered. A number of reviews on this subject are available (*7–13*). The clinical implications of particulate matter in injections are determined by many factors, including the size and number of particles, the composition of the material, the potential for microbiological contamination, the route of administration, the intended patient population, and the clinical condition of the patient. For example, an otherwise healthy individual receiving a subcutaneous or intramuscular injection containing sterile, inert particulates would likely experience no adverse effect or at worst would develop a small granuloma. On the other hand, a critically ill premature infant receiving a particle-laden infusion directly through an umbilical catheter might suffer considerable pathophysiologic sequelae (*14,15*).

Garvin and Gunner were among the first to report a concern about the effects of particles in human patients (*16,17*). For obvious ethical reasons, there is a lack of controlled clinical studies on the effects of particles in human patients. Some anecdotal information about human patient safety may be obtained by examining case reports of intravenous drug abusers (*18–20*). In these cases, solid oral dosages are often ground up and injected as a slurry; pulmonary foreign body emboli and granulomas were observed in these patients (*21*). Unfortunately, the clinical risks to human patients posed by small numbers of particles are difficult to infer from these observations due to the extreme number of insoluble particles and the uncontrolled conditions in which they were administered.

Numerous animal studies have been conducted to determine the fate of intravenous particles with different sizes and composition (*22–25*). Most studies have focused on subvisible particles with a diameter of <50 µm. In these studies, a massive infusion of particles has been accompanied by histologic evidence of injury to pulmonary capillary endothelial cells (*26*), microscopic thrombi in the pulmonary capillaries (*27*), pulmonary microscopic granulomata (*28*), and hepatic inflammatory effects (*29*). Although useful for understanding the pathophysiologic response to particulate matter, the large number of particles used in these studies (e.g., 10^9 particles/kg per injection) provides little insight into the risk to humans posed by small numbers of macroscopic particles. Arterial embolization using materials such as polyvinyl alcohol (PVA), collagen-coated acrylic microspheres, and gelatin spheres also provides some insight into the potential human pathophysiologic implications of non-target embolization of extraneous-particle intravenous infusions. In these cases, massive particle loads moving from the arterial injection site into the venous circulation were also reported (*30–34*).

In a review of the hazards of particle injection, it has been found that the primary contributor of particulate matter in vials is the rubber closure due to coring, a risk that is present with almost every injection from this presentation. In addition, case reports have documented injury associated with infusion of significant quantities of precipitated admixtures or therapeutic use of particles for embolization (*14,15,35*). Despite the administration of an estimated 15 billion doses of injectable medicines each year (*36*), no reports of adverse events associated with the injection of individual visible particles have been found.

Ultimately, the safety considerations related to particulate matter in injections must be assessed for each drug product, intended patient population, and method of administration. No single set of inspection criteria can adequately anticipate all of the potential risks to the patient. The methods outlined in ⟨790⟩ should serve as essential requirements when assessing the adequacy of the visual inspection procedure, but alternative acceptance criteria (for example, the use of tightened sampling plans) should be implemented when the patient population and intended use of the product warrant these additional measures.

2.3 History of Compendial Inspection Standards

The requirement for injections to be "true solutions" appeared in *USP IX* in 1915, and the first appearance of "solution clarity" for parenterals occurred in 1936 in *NF IV*. Since then, there have been numerous modifications to the compendia in this regard. A comprehensive history of compendial inspection standards is available in the *Pharmacopeial Forum* (*37*).

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3. TYPICAL INSPECTION PROCESS FLOW

3.1 100% Inspection

Chapter ⟨790⟩ establishes the expectation that each unit of injectable product will be inspected as part of the routine manufacturing process. This inspection should take place at a point when and where defects are most easily detected; for example, prior to labeling or insertion into a device or combination product. Each unit may be examined manually with the unaided eye, or by using a conveyor to transport and present the containers to a human inspector (semi-automated inspection), or by means of light obscuration (LO) or electronic image analysis (automated inspection). Manual and semi-automated inspection should only be performed by trained, qualified inspectors. Inspection may also be enhanced by means of a device that holds more than a single unit at one time for examination. This inspection may be performed at-line or in-line with filling or packaging or in a separate, off-line inspection department. The intent of this inspection is the detection and removal of any observed defect. When in doubt, units should be removed (see *Figure 1*).

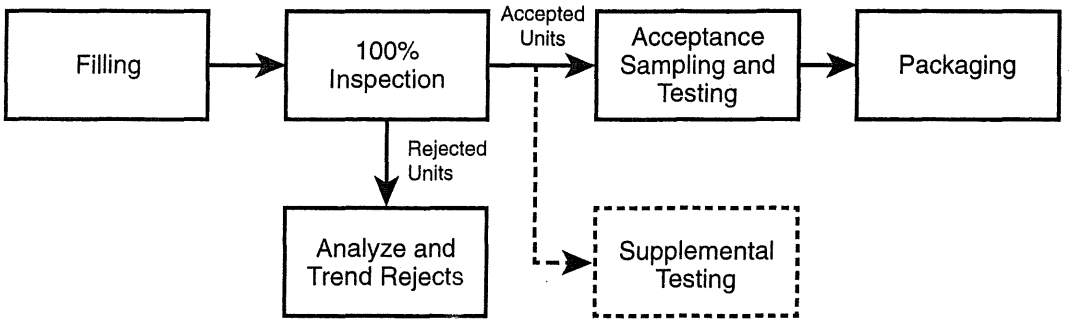

Figure 1 shows a simplified process flow. Alternative strategies, such as reinspection or two-stage inspection, may be required and are discussed in *3.3 Remediation and Alternative Practices*.

[NOTE—100% inspection refers to the complete inspection of the container–closure system and its contents. Inspection may be accomplished in a single operation or in multiple steps using a combination of technologies. See additional discussion in *3.3 Remediation and Alternative Practices* and *6. Inspection Methods and Technologies*.]

[NOTE—Supplemental testing is required when the nature of the product or container limits visual inspection of the contents (e.g., with a lyophilized cake or powder, or with an amber glass or opaque container). See additional discussion in *5.2 Unique Product and Container Considerations*. Samples for supplemental testing may be taken from any point in the process after 100% inspection.]

During 100% inspection, limits on typical rejection rates should be established to identify atypical lots (*38*). These limits may be established for categories of defects (e.g., critical, major, and minor) or for specific types of defects (e.g., particles). A review of historical performance is useful in establishing these limits, and the review may include grouping products similar in appearance and manufacture. Periodic reassessment of these limits is recommended to account for expected process improvements and/or normal fluctuations in process baseline (*39*). If a limit is exceeded, it should trigger an investigation. The investigation may include additional inspection or it may determine whether additional inspection is necessary.

3.2 Acceptance Sampling and Testing

After 100% inspection, a statistically valid sample is taken from the units accepted by the inspection process. These sampled units should be manually inspected under controlled conditions by trained inspectors. Chapter ⟨790⟩ provides reference inspection conditions for this purpose. The sample may be a random or a representative sample (e.g., at fixed time intervals or a fixed number per tray). Defects may not be distributed equally over the lot, and therefore a sampling process that represents the whole lot is required. Typical sampling plans used for this purpose can be found in the ANSI/ASQ Z1.4 standard (*40*). Equivalent plans may also be found in the ISO 2859 (*41*) or JIS Z9015 (*42*) standards. For batch release, the sampling plans listed as Normal II are typically used. Tightened sampling plans may be appropriate when an atypical result is observed or reinspection is performed. These plans specify a sample size for a range of batch sizes and require selection of an acceptable quality limit (AQL). The AQL is the defect rate at which 95% of the lots examined will be accepted and is a measure of falsely rejecting good batches. Critical defects (those that pose the greatest risk to the patient) should be assigned an AQL with a very low value. Often, the accept number (the number of defective units allowed in the sample) for a critical defect is zero. Major and minor defects, which pose less risk to the patient, will have increasing (less stringent) AQL values and accept numbers greater than zero. Further definition and discussion on defect catagories is found in *5.1 Defect Classification*. *Table 1* shows the range of AQL values typically used for visual inspection processes (*43*).

Table 1. Typical AQL Values for Visual Inspection Processes

Defect Category	AQL Range (%)
Critical	0.010–0.10
Major	0.10–0.65

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Table 1. Typical AQL Values for Visual Inspection Processes *(continued)*

Defect Category	AQL Range (%)
Minor	1.0–4.0
[NOTE—When selecting a sampling plan for AQL testing after 100% inspection using ANSI/ASQ Z1.4, ISO 2859, or JIS Z9015, choose the sample size to satisfy the AQL value for the most critical category (e.g., critical) of defects being evaluated. Then use the accept numbers for this sample size for the AQL values chosen for the other defect categories (e.g., major and minor). This assures that the sample size will produce a statistically valid result for all defect categories examined. The defect categories shown here represent a common basic approach to grouping defects by risk; however additional categories may be added to these for more detailed analysis.]	

While the standards referenced in the paragraph above are indexed by AQL, it is important to also know the unacceptable quality limit (UQL) for the sampling plan(s) used. These can be found in the operational characteristic (OC) curve data supplied for each plan found in these standards or calculated independently using qualified software. The UQL for the sampling plan used should also be known. The UQL is the defect rate at which 90% of the lots examined will be rejected and is a better measure of the customer or patient risk. The protection afforded by any sampling plan is represented by its OC curve. This is a plot of the probability of lot acceptance versus the defect rate in the lot. The AQL and UQL are two points on this curve. Sampled units should be manually inspected under controlled conditions by trained inspectors. Inspection conditions should be aligned with the 100% inspection process.

Manual acceptance sampling should be performed after any type of 100% inspection process, including manual, semi-automated, and automated inspection processes. It provides a measure of the performance of the overall inspection process and the quality of a specific lot, compared with predefined acceptance criteria. Although automated systems are validated before use and are routinely challenged to ensure acceptable performance, the use of manual acceptance sampling inspection detects unexpected defects that were not included in the development and training of the automated system by the manual inspection process.

Acceptance criteria are comprised of the product specifications and acceptance/rejection criteria, such as the AQL and UQL values, with an associated sampling plan that is necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units) as described in 21 CFR 210.3 (*44*). If the acceptance criteria of the sampling plan are not met, an investigation should be conducted. Depending on the nature of the failure, this investigation should include forensic classification/identification of the particle, and examinations of the manufacturing process, the raw materials, and the packaging materials, as well as the inspection process. If, after investigation, the inspection process is deemed capable of detecting the defect(s) in question, the batch may be reinspected. An alternative inspection process, better suited to detection of a specific defect may also be chosen for reinspection. After reinspection (performing a second 100% inspection of the batch), a new sample of the accepted units is taken and compared against established acceptance criteria. It is a good practice to use a tightened sampling plan and acceptance criteria under these circumstances because of the atypical nature of this process step.

3.3 Remediation and Alternative Practices

REINSPECTION

As discussed in the preceding section, reinspection (repeating the 100% inspection followed by acceptance sampling inspection) may be appropriate if the initial 100% inspection is not successful. This includes instances when the established 100% inspection failure rate(s) and/or the accept/reject number(s) associated with the chosen AQL values have been exceeded. Reinspection should only be conducted using a procedure that has been approved by the quality organization and addresses key parameters such as the inspection conditions (e.g., same as primary inspection or modified to enhance detection of a specific defect type), the number of times reinspection may be performed (this should be limited and justified), and the acceptance criteria (e.g., same as primary inspection or tightened). If reinspection is required often, consideration should be given to improving the sensitivity of the primary inspection process or of the manufacturing controls as determined by root cause analysis.

TWO-STAGE INSPECTION

In cases where an assignable cause, such as formation of air bubbles or specific container or closure variation, results in a high false-rejection rate (rejection of acceptable units), the use of a second inspection step may be considered. *Figure 2* shows a typical process flow for a two-stage inspection. Such an inspection strategy is more common with automated inspection systems, where there is less ability to tolerate normal variation in product or container. Under these circumstances, the inspection system is adjusted to ensure acceptance of good units. Those not accepted are considered of uncertain disposition until inspected by another means (e.g., manual inspection following automated inspection) to provide confirmation of acceptance or rejection. Inspection conditions may be adjusted to provide greater sensitivity in this second inspection step (e.g., additional inspection time) to ensure a high probability that true defective units will be rejected. The limitations of the first inspection and the reason for conducting a second stage of inspection should be clearly defined and documented. The second inspection of these units by the same method and conditions (e.g., automated inspection with the same parameters after an initial automated inspection) is generally not recommended because the same limitation in inspection method is present for both inspections. However, it may be suitable when the root cause is air bubbles in the solution and a study has been performed to establish an appropriate holding time to allow the bubbles to dissipate before performing the second inspection. It is recommended that each inspection stream (those accepted by the first stage and those accepted by the second stage) be sampled separately and evaluated against the sampling plan acceptance criteria before they are confirmed as accepted and recombined into a single batch.

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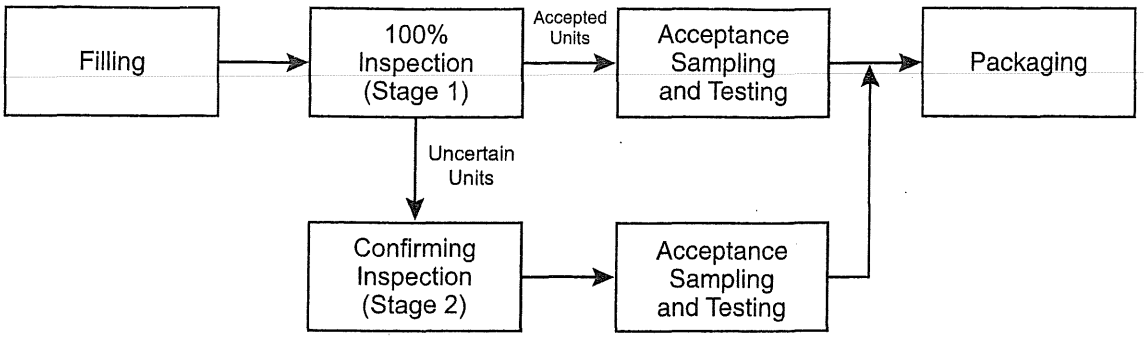

Figure 2. Two-stage inspection process flow chart.

If a two-stage inspection strategy is used, it must be validated as intended for use. Defective containers with less than a 100% PoD will have the PoD reduced further with each stage of inspection, thus the PoD should be determined after inspection through both stages to ensure that acceptable sensitivity is maintained.

4. INSPECTION LIFE-CYCLE

4.1 Extrinsic, Intrinsic, or Inherent Particles

Particles may originate from many sources. These are discussed here, as well as in other chapters in the *USP* (e.g., ⟨1787⟩). Those that are foreign to the manufacturing process are considered to be exogenous or "extrinsic" in origin; these include hair, non-process-related fibers, starch, minerals, insect parts, and similar inorganic and organic materials. Extrinsic material is generally a one-time occurrence and should result in the rejection of the affected container in which it is seen; however, elevated levels in the lot may implicate a broader contribution from the same source. These particles may carry an increased risk of microbiological or extractable contamination, because less is known about their path prior to deposition in the product container or their interaction with the product.

Other particles are considered "intrinsic", from within the process, or "inherent", which are known to be or intended to be associated with specific product formulations. The determination of whether the particulate is inherent or intrinsic to the process is based upon appropriate characterization of the particle's physicochemical properties. Intrinsic particles may come from processing equipment or primary packaging materials that were either added during processing or not removed during container preparation. These primary product-contact materials may include stainless steel, seals, gaskets, packaging glass and elastomers, fluid transport tubing, and silicone lubricant. Such particles still pose the risk of a foreign body, but generally come from sterile or sanitized materials and more is known about their interactions when in contact with the product. Any process-related intrinsic particles should have controls established based on the use of a life-cycle approach as outlined in *1.3 Defect Prevention*. Another group of particles considered intrinsic is interrelated with the stability of the product. These product stability-related particles come from container–closure interaction, changes to the drug formulation (insoluble degradation products), or temperature sensitivity over time. Stability-related intrinsic particles should be identified and addressed as early in the product development process as possible.

The physical form or nature of inherent particles varies from product to product and includes solutions, suspensions, emulsions, and other drug delivery systems that are designed as particle assemblies (agglomerates, aggregates). Product-formulation-related particulate formation should be studied in the development phase and in samples placed on stability to determine the normal characteristics and time-based changes that can occur. Use of automated particle counting or image analysis in the subvisible (for particle sizes ≥2 µm) and visible ranges may be required to fully characterize inherent formulation-related particles. In biologics, protein particles are considered inherent and may be accepted when their presence may be measured, characterized, and determined to be part of the clinical profile. Inherent particles may be accepted if the drug product has a control strategy showing that this particulate category is part of the product clinical profile. The manufacturer may allow inherent particles if the product appearance specification also allows their presence or if the product is an emulsion or suspension.

An evaluation of the potential impact of particles identified from any of these sources may be enhanced by incorporating a clinical risk assessment. This assessment may include factors such as the intended patient population, route of administration, source of the particles, and implications for product sterility. For intrinsic or inherent particulate matter sources, a risk assessment may be useful in developing product-specific control strategies. Given the probabilistic nature of particle detection, it is important to assess the possible implications of particles identified through the product life-cycle to better ensure the product's safe use.

4.2 Prevention of Particulates

The manufacturing process is designed to keep the final container and its contents clean within the control parameters established for process-related particulates. Once the container is filled, the stability of the product needs to be maintained throughout its shelf life. Changes that occur as the product ages during its normal shelf life must be characterized. Avoidance of intrinsic particle sources that may affect final product stability depends on careful consideration of the entire product system. If these intrinsically sourced changes occur, and they affect stability, particles ranging from subvisible to visible may develop. Typically, these particles result from change mechanisms that slowly affect the on-shelf product.

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ROBUST DESIGN DURING DEVELOPMENT

To anticipate potential sources of instability that yield intrinsic particles, the product design should be evaluated from many perspectives, beginning with a literature review of similar formulae/packages. Points to consider include the reported sensitivities of the active ingredient, the formulation type, and the final container–closure system needed for delivery. Knowledge of how glass containers are fabricated, controlled, sterilized, and tested is important as this may affect the tendency to form glass lamellae as discussed in *Evaluation of the Inner Surface Durability of Glass Containers* ⟨1660⟩ and by the FDA (*45*). Obtaining further information on residual extracts, possible leachates, metals, or solubility-edge conditions is important as these factors may promote formation of solid material in the aging solution. Several additional key factors for successful product design include the product concentration, solution pH, critical micelle concentration, oligomerization content/potential, package effects (large surface area, product volume, headspace, light/oxygen transmission), and compatibility of the formulation with the package. Furthermore, shock/vibration sensitivity at the air/liquid interface can be a significant contributor to particle formation that requires investigation during product development. Some key formulation design factors include the formula components chosen and their purity; the solubilities of the active ingredient(s) and excipients, and consideration of potential salt forms. Finally, to maximize product stability, consider the final product preparation for delivery, product dilutions, and shelf stability of the commercial product or its therapeutic preparations.

To examine the appropriateness of the product design for maintaining product stability, there are two levels of evaluation. Both levels examine retained containers for visible changes using methods described in this chapter, but neither level dwells on low percentage defects.

For the first level of stability study, bench trials consisting of visual inspection of trial containers in the formulation lab will show general compatibility of the chosen components over time with regard to clarity, color, and particle formation. Careful product assembly in clean containers, with consideration of the container type, headspace, and sealing, will yield a beneficial first-pass trial of stability over several months' time. Particles will be detected, and the investigation of type is essential in order to differentiate additive types from instability or package interation. Pursuit of extrinsic particles at this stage of development is generally not significant, as the particles do not reflect on the formulation or manufacturing process that is under development, but rather the manner in which they were filled.

The second, more refined level of stability study involves conducting visual inspections of the injection in defined, International Council for Harmonisation (ICH)-relevant trials (*46*). This may include periodic inspection of the same containers over time if the product does not require reconstitution or is not affected by frequent temperature changes. Detection of minor or subtle differences in these containers is not the goal at this stage of development. Catastrophic change and the occurrence of intrinsic product-related visible particles should be the focus. Typically, a set of containers is carefully prepared to exclude extrinsic particles and is then inspected to cull out any units with visible defects. Next, a numbered set of containers appropriate for the batch size is placed on trial and visually inspected periodically for changes due to instability. It is important to be able to analyze the particulate matter or condition (color, haze) in order to identify the cause of truly stability-indicating events. A typical sample size is 80–100 units. This should be a sufficient trial of the package and formulation interaction. Additional sets of containers stored at selected extremes of ICH temperatures can be followed to aid discovery of solubility-edge phenomena. When unwanted changes are detected, such as particle formation, solution color change, solution haze, and package changes, the process of isolation, characterization, and identification can commence. Identification of the material making up the changes aids in determination of the cause, as well as development of improvements for future use.

COMMON SOURCES OF INTRINSIC PARTICULATES

Process-related intrinsic particles originating from product contact materials tend to be stable and unchanging (e.g., glass, rubber, or metal). In contrast, there may also be particles resulting from product stability-related change mechanisms within the final product. It is very important to understand that these changes only have to be slight in certain cases, far below the detection limit of most release or stability assays, to result in visible changes to the product. The threshold levels for the formation of visible change for certain substances may be only 10–100 ppm (0.001%–0.01%) based on the ability to detect a single 100-µm particle or many sub-10 particles giving a hazy or cloudy appearance. However, if all of this insoluble material were contained in a single visible particle, it would likely cause rejection of the container.

FORMULATION COMPONENTS

The active ingredient may also contribute to the presence of stability-indicating intrinsic particles. For example, significant haze and particles have manifested in aqueous formulations due to extraction of plasticizers from filtration media during bulk drug production (*5*). Metal content in the active ingredient has contributed to organometallic salt formation and has also been observed as precipitated inorganic salts, blooming long after product release. The active ingredient and related degradation products may also be relatively insoluble and may grow to form visible particles. The particulate material must be analyzed to determine its chemical nature and possible identification.

Monomers or single molecules may join together through chemical processes to form dimers, trimers, and oligomers (a limited assemblage of monomers, short of polymerization). Such changes are not unexpected (*47*). In high-concentration and/or saturated formulations, and especially for micellar drug associations, the solubility of related forms is significant when the aging formulations contain progressively higher concentrations of these substances. Larger molecules may have a greater effect on solution integrity due to their inherent insolubility, especially if the active drug is in a micellar formulation.

Polymorphs are unique crystalline forms of identical chemical entities. Although uncommon in solutions that have been mixed homogeneously and filtered, small seed crystals of a relatively stable polymorph may form over time, especially at nucleation sites such as container-surface defects. More common than formation of polymorphs is formation of a modified crystal lattice containing an integral liquid, typically water or solvent. The lattice may form slowly, promoted by evaporation, nucleation, and temperature extremes (*48,49*).

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PACKAGING COMPONENTS

Extractables and leachables are terms commonly used to describe the potential for primary packaging materials to contribute unwanted agents to the product. Extractables represent all of the materials that could be contributed, and leachables represent the practical contribution upon contact between packaging components and drug formulation (*50*). These substances can also contribute to the formation of subvisible and visible particles.

Formulation attack of the container is a dramatic change and most often occurs in glass container systems. Glass containers undergo corrosion that is 25 times greater at pH 8 than at pH 4 (*51*). A formulation pH above 7, especially with high-ionic strength solutions, promotes attack of the inner glass surface, resulting in particle generation.

Silicone oil is added to pre-filled glass syringe systems to enhance lubricity for stopper or plunger insertion and its movement within the syringe barrel. Silicone may also come from tubing used for fluid transfer and a variety of polymeric fittings and seals that are used in the processing equipment. All of these components must be compatible with the formulation to minimize leachates. Although silicones are processed to be sterile and are widely used, their use must still be controlled. Silicone can cause container sidewall droplets and a variety of visible semi-solid forms. No more than the minimum quantity should be used during processing. Silicone and other hydrophobic substances have the capacity to coalesce and agglomerate with other particles, reaching a visible size.

4.3 Particulate Removal by Component Washing

GLASS CONTAINERS

Each step of the glass-container washing and rinsing process should be evaluated for particle-reduction capability. The washer validation studies should demonstrate a reduction in naturally occurring particles or should use seeded containers to demonstrate such reduction capability. The use of statistical sampling plans with LO and/or membrane microscopic particle-counting methods can provide a means to demonstrate reduction of both subvisible and visible particles during washing cycle development and validation. The membrane filtration microscopic method is superior to LO methods for capturing and characterizing larger foreign particles in the visible range (>100 µm) during validation or monitoring activities. During process development, validation, and routine use, container-washing procedures should include periodic visual operational checks. This routine verification ensures that effective draining of all containers is occurring during all washing and rinsing steps. Review the wash-water recirculating filter maintenance procedures to ensure that particle overloading or breakthrough is being prevented.

Glass breakage that occurs during the component washing process could affect surrounding containers and the washing cycle should be evaluated for possible glass particle generation and distribution. Effective, written container-clearance procedures following these occurrences should specify the number of containers to be removed from the affected portion of the line. Removing units that could potentially contain glass particles aids in minimizing particle transfer to the downstream process.

ELASTOMERIC CLOSURES

Each step of the elastomeric-component washing and rinsing process should be evaluated for particle-reduction opportunities. Utilize statistical sampling plans to collect meaningful test units. LO or other automated particle counting and membrane microscopic particle-counting methods may be used to demonstrate reduction of both subvisible and visible particles during washing validation. The membrane filtration microscopic method is superior to LO methods for capturing and characterizing larger foreign particles in the visible range (>100 µm) during validation or monitoring activities. During process development and validation and in routine use, container-washing procedures should include visual checks to ensure that stoppers are not routinely sticking together. Such sticking surfaces reduce cleaning efficacy and entrap particles. Periodic assessment of component cleanliness and supplier washing capabilities should be included as part of the supplier qualification program when using purchased, ready-to-sterilize, or ready-to-use components.

Evaluate any current siliconization process used, whether in-house or by the supplier, to minimize excess silicone levels while maintaining machinability of the stoppers. LO or other automated particle-counting method may be used to compare overall particle level reduction (background silicone oil droplets) during process development or validation. The level of residual silicone oil will affect the particulate quality of the final filled product, observed as dispersed droplets and particle-forming matrices.

GLASS HANDLING

Processes that use racks or trays for transporting and holding samples, as are typically used in batch ovens, should be monitored for metal particle generation. The racks or trays should have a formal maintenance program associated with their routine use. Trays should be inspected for wear and scoring, which can be sources of particulates. Periodic cleaning, polishing, and/or resurfacing may be warranted to effectively control particles. Tunnels used for depyrogenation should also have a routine maintenance program for periodic cleaning, inspection, and replacement of parts that may wear and generate particles. Routine process observation for glass breakage allows for clearance of any potentially affected surrounding containers and minimizes the occurrence of glass particles being carried downstream to filling. Glass-to-glass and glass-to-metal contact should be minimized where possible to reduce weakening of the glass surface with increased risk of subsequent fracture. The use of polymeric facing on guides can help in reducing such damage.

EQUIPMENT PREPARATION

It is important to minimize redeposition of particles on product contact surfaces after cleaning. Cleaned and sterilized equipment should be protected by HEPA-filtered, unidirectional airflow until transferred to, and installed on, the filling line. For cleaned equipment that needs to be wrapped or bagged prior to sterilization, utilize low-shedding, noncellulose (synthetic)

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wrapping materials. Cellulose fibers are one of the most common particles found in the injections-manufacturing environment and injectable products and their origin will be a prime concern (*43*).

Personnel are a concern for introduction of extrinsic particle types such as eyebrows, eyelashes, facial hair, and skin cells. The equipment preparation staff should be adequately gowned with hair covers, facial hair covers, and goggles to prevent contaminating cleaned process equipment.

During validation and monitoring activities of clean- and/or steam-in-place (CIP-SIP) systems (vessels, filters, tubing, and other product contact equipment) foreign particle burden should be evaluated after cleaning.

FILLING LINE

The transfer of open containers should be evaluated and reviewed to mitigate particle contamination. For example, for aseptically filled products the transfer should be conducted in Grade A (ISO 5, Class 100), unidirectional air flow to minimize particle contamination. The air in critical zones should be monitored continuously during operation to confirm compliance.

Routine checks to detect particles and potential particle-generation locations should be explained in the procedures. Effective, written container-clearance procedures to be used after glass breakage should specify the number of containers to remove from the affected portion of the line. Note that improper set-up and adjustment of the filler can lead to "needle strikes" where the filling needles make contact with the container being filled. This can generate either stainless steel or glass particles.

Filling pump design and the pump's compatibility with the filling solution are important considerations. Metal-on-metal piston pumps have a greater potential for generating metal particles, compared with other types of piston pumps. Pump maintenance is essential and includes a requirement to resurface the cylinders and pistons periodically. Peristaltic-action pumps must be monitored for generation of silicone tubing particles, especially with aggressive, near-saturated solutions or suspensions. Friction in the peristaltic roller area can break down the tubing, resulting in the generation of particles (spallation).

Stopper bowl surfaces should have a formal maintenance program, and stopper handling or replenishment by operators should be specifically designed to minimize particle transfer to the stoppers. Proper operator positioning and avoidance of open containers is important in good aseptic-filling practices to avoid microbial contamination. These same principles help reduce particle transfer to the open containers and exposed elastomeric closures.

Careful selection of cleaning and gowning materials will help reduce contamination from extrinsic particles and fibers. These clean-room materials should be selected for their superior nonshedding and low-particle properties.

4.4 Trending

Data obtained from the in-process 100% inspection followed by AQL inspection are used for batch release. Both 100% and AQL inspection data should also be analyzed for adverse trends on a periodic basis, typically at least annually. Data from the 100% inspection provides the best source of typical defect types and rates during normal production. High-volume products may generate sufficient data to allow quarterly analysis, whereas a longer period of time may be necessary to accumulate data for products that are produced infrequently. Data from component inspection, production 100% inspection, and the AQL inspections should be evaluated based upon sound statistical principles to determine whether the current action levels are accurately reflecting the current process capability. Alert and/or action levels may be established and/or adjusted if the statistical analyses indicate that lower defect levels are being observed consistently.

When establishing new action or alert levels, a preliminary value may be used until sufficient production experience is obtained. Consideration should be given to planned improvements in the manufacturing and inspection processes. If significant improvements are planned, the reduction of the action/alert level should not be instituted until the impact of the improvement is measured over sufficient time to establish the validity of the new value.

5. INTERPRETATION OF INSPECTION RESULTS

5.1 Defect Classification

Defects are commonly grouped into classifications based on patient and compliance risk (*1*). The most common system uses three groups: critical, major, and minor. Critical defects are those that may cause serious adverse reaction or death of the patient if the product is used. This classification includes any nonconformity that compromises the integrity of the container and thereby risks microbiological contamination of the sterile product. Major defects carry the risk of a temporary impairment or medically reversible reaction, or involve a remote probability of a serious adverse reaction. This classification is also assigned to any defect which causes impairment to the use of the product. These may result in a malfunction that makes the product unusable. Minor defects do not impact product performance or compliance; they are often cosmetic in nature, affecting only product appearance or pharmaceutical elegance.

Upon 100% inspection, visible extrinsic and intrinsic particles should be reliably removed. Particle motion aids in detection, and stationary particles are difficult to detect, exhibiting a significantly reduced PoD. The test method allows inherent particles to be accepted if the product appearance specification allows inherent particle types. The size of particles reliably detected (≥70% PoD) is generally 150 µm or larger (*4*). Fibers of similar length are generally detected with a lower PoD due to their smaller cross-sectional area and varied orientation during inspection. PoD for all particles is dependent on the container characteristics (e.g., size, fill level, shape, and transparency), inspection conditions (lighting and duration), formulation characteristics (color and clarity), and particle characteristics (size, shape, color, and density). The PoD at 70% or greater is known as the "reject zone" described in Knapp's methodology (*52,53*), which is used worldwide as an industry common practice for rejecting particle defects. Test sets characterized by repeated inspections, as described in *7.4 Rejection Probability Determination*, are used to "calibrate" the inspection method's PoD, inspector performance, or automated inspection systems, and to demonstrate the sensitivity to threshold particle size at the reject zone of >70% PoD. It should be understood that the limitation of the reject zone at 70% detection is that at this size threshold particles of the same size may routinely be missed or

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go undetected up to 30% of the time. These undetected units may contain some amount of threshold-sized particles or subvisible particles at a lower PoD. It is therefore important to characterize any particles recovered from AQL testing, retention sample inspection, and product returned from distribution to understand how they could have gone undetected originally during the initial 100% in-process inspection.

5.2 Unique Product and Container Considerations

LYOPHILIZED PRODUCT

Lyophilized products receive 100% inspection after the freeze-drying step has been completed and each unit has been sealed. However, the solid, lyophilized cake can mask the presence of visible particles because they cannot be seen within the solid matrix. The cake surface is visible during inspection but accounts for only a small fraction of the cake volume. Because of these challenges in evaluating acceptability, a small sample of units is reconstituted and inspected for visible particles in addition to the 100% inspection of the cakes for visible particles. Care must be taken during reconstitution of these samples to avoid contamination that can lead to false-positive results. Sample preparation should be done in a clean environment with appropriate particle-control measures. Reconstituted samples should be inspected using the same conditions as those for visible particles. The destructive nature of this test limits the size of the sample; however, the resultant fluid allows visible particles to be more readily detected. Typical sampling plans for this type of test can be found in the special sampling plans S-3 and S-4 in ANSI/ASQ Z1.4 (*40*). The S-plans offer a practical compromise between sample size and statistical power and for most batch sizes between 3,201 and 150,000 suggest a sample size of 20 with an accept number of 0 (based on an AQL of 0.65%). Sample sizes larger than 20, as found in these sampling plans, may be appropriate for larger batch sizes when additional sensitivity is desired. Alternative plans are acceptable, but care should be taken to examine the UQL of such plans to assess their sensitivity. Once inspection of these reconstituted samples has been performed, they may be used for other required testing, such as that for subvisible particles, potency, impurities, or other specified tests. If particles are detected in this relatively small sample, additional units may be reconstituted as part of an investigation and to assess the compliance of the entire batch. The size of the additional sample should be based on the total sample size (initial plus additional sample) required to have an accept number greater than 0 for a sampling plan with an AQL of 0.65% or less. This will be based on the batch size. The results from the samples must be combined, rather than resampling and basing the accept decision on the results of the second sample only.

Alternatives to reconstitution can be considered, such as collection of lyophilized samples from the filling line after stopper insertion. Such samples represent the majority of particle exposure risk. These risks should be assessed and documented to justify this approach.

POWDER PRODUCT

Sterile powders are difficult to inspect for particles due to powder flow and the occlusion of white or light-colored particles by the drug product itself. Sterile powders should be reconstituted and inspected for visible foreign particles using an approach similar to that for lyophilized products, as discussed above.

EMULSION AND SUSPENSION PRODUCT

The manufacturer may allow inherent particles if the product is an emulsion or suspension. For suspension products, a test dissolving the suspension or disruption of the emulsion that provides for extrinsic and intrinsic particle detection is also recommended as part of destructive supplemental testing of a small sample as described above for lyophilized products.

AMBER CONTAINERS

Inspecting amber containers is challenging because selected elements have been added to mask UV light penetration into the Type I glass container. Light transmission is blocked below 500 nm, and thus increased light intensity (e.g., 8,000–10,000 lux) may be required to observe visible particles during inspection. Directional lighting from behind the container may also be beneficial. At the extreme, filled solution in practically opaque containers may be audited via sampling and transferred to clear, clean containers. The membrane filtration microscopic method is also suitable for capturing and characterizing larger foreign particles in the visible range (>100 µm).

TRANSLUCENT PLASTIC CONTAINERS

Translucent plastic containers are chosen for break resistance or other properties that glass cannot offer, such as injection molding into shapes that minimize hold-up volume or for use in a combination product. Plastic containers may have optical properties that require significantly more light (e.g., 8,000–10,000 lux) to illuminate any visible particles against black and white backgrounds. Directional lighting from behind the container may also be beneficial.

LARGE-VOLUME CONTAINERS

Large-volume containers (>100 mL) may require additional time to complete a thorough inspection. For flexible bags, the semitransparent nature of the PVC film used to manufacture these containers may require the use of additional light intensity to enhance the visibility of particles. Directional lighting from behind the container may also be beneficial.

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COMBINATION PRODUCTS

When inspecting the unlabeled primary drug container for a combination product, the inspection considerations should be the same as those specified for a conventional drug product in a vial or syringe. This inspection should be performed before assembly into the device. Where there are critical attributes that are only visible after assembly (such as alignment with a fill-level window), a second inspection after assembly may also be required.

5.3 Alternate Inspection Strategies for Supplemental Testing

TRANSFER

When the container limits thorough inspection, transfer to a clear and readily inspectable container is recommended. Using verified clean and verified clear containers, the sample is opened and drained to the receiving container, plugged, and then visually inspected.

FILTRATION

Membrane filtration methods, such as in *Particulate Matter in Injections* ⟨788⟩, *Method 2 Microscopic Particle Count Test*, collect all solid particles from the fill onto a membrane. Samples may be individual or pooled for analysis. This method will reveal all solid particles (visible and subvisible), which may be sized microscopically, and permits qualitative categorization of these retained solids.

CLARIFICATION

In the case of suspensions, there is a wide range of active ingredient particle sizes, from nano–sized (<1 µm) to tens of micrometers. In all cases, the solids may be clarified/dissolved in the original container with an appropriate filtered solvent to allow subsequent visual inspection. Solvent compatibility with the formula and package must be demonstrated.

SIEVING

As above, if the solid particle suspension is small enough to allow selective sieving, this may be used as an alternative to membrane filtration. The very small solids pass through the sieve and larger (>100 µm) particles are retained, counted, and categorized.

6. INSPECTION METHODS AND TECHNOLOGIES

6.1 Manual Visual Inspection

Manual visual inspection (MVI) is the reference inspection method described in all of the major pharmacopeias (*54*,*56*). It consists of viewing filled and sealed containers under controlled conditions. This process may be aided by the use of a tool to allow consistent examination of more than one container at a time. The quality decision, to either accept or reject the container, is made by a trained person. Inspection is a probabilistic process, and detection rates <100% are to be expected, especially for smaller or low-contrast defects.

CRITICAL PROCESS PARAMETERS IN MVI

Light intensity: The results of the manual inspection process are influenced by the intensity of the light in the inspection zone. In general, increasing the intensity of the light that illuminates the container being inspected will improve inspection performance; ⟨790⟩ recommends light levels NLT 2,000–3,750 lux at the point of inspection for routine inspection of clear glass containers. This range was chosen to harmonize with current *European Pharmacopoeia* requirements (*55*) and is further supported by recent studies performed in Japan (*55*) as part of a review of the current *Japanese Pharmacopoeia* requirements (*56*). Special attention should be given to ensure that inspection is not performed below the lower limit of 2,000 lux. Increased light levels are recommended for translucent plastic containers or those made from amber glass. Under these circumstances, light levels as high as 10,000 lux may prove beneficial. Care should be taken to avoid glare and direct viewing of the light source at these high intensities, as this may result in eye strain and fatigue. The final inspection conditions will depend on measured performance.

Light should be diffuse and even across the inspection zone, and it is a good practice to clearly identify this zone within the inspection station where the intensity meets the required levels. Fluorescent lamps have often been used as the light source for inspection. When fluorescent lamps are used, high-frequency ballasts are recommended to reduce visible flicker (and associated inspector fatigue). Incandescent lamps have also been used successfully for this purpose, but they generate significant heat during use. Light-emitting diodes (LED) offer an energy-efficient, stable source of light without the added heat of incandescent lamps.

Light intensity in each inspection station should be measured periodically to ensure continued compliance within the specified range. The frequency of monitoring should be based on historical experience with the type of light source in use. A lower light-intensity action limit should be established to trigger corrective action before inspection is performed below the lower limit of the range.

Background and contrast: Contrast between the defect of interest and the surrounding background is required for detection, and increased contrast improves detection. The use of both black and white backgrounds is described in ⟨790⟩, as

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well as other global pharmacopeias. Matte/nonglossy backgrounds are recommended to avoid interference from reflection. The use of both backgrounds provides good contrast for a wide range of particulate and container defects, which can be light or dark in appearance.

Inspection rate: Sufficient time must be provided to allow for thorough inspection of each container; chapter ⟨790⟩ specifies a reference time of 10 s per container (5 s each against both black and white backgrounds). Larger or more complex containers may require additional time for inspecting all attributes. Increased time may facilitate detection of defects near the threshold of detection, but studies by Wolfe, et al. (*57,58*) suggest that there are diminishing gains with increasing inspection time. Time spent per container may be controlled through the use of a pacing device such as a light or tone, or these may be used during training only, much as a musician uses a metronome during practice to learn the tempo of a musical piece for later performance. Recording the time spent inspecting each batch and then calculating a nominal inspection rate is a good way to confirm that the rate of inspection was within established limits. Correction can be made for noninspection activities performed during this time by the inspectors to better document the nominal inspection rate.

Container handling and movement: When observing objects, the human eye is very sensitive to movement. Good techniques for manual inspection include a careful swirl or inversion of the liquid product within the container. This displaces any particles from the upper inner surfaces of the container and the closure and puts them into motion. A technique that minimizes the introduction of air bubbles is important, as air bubbles can appear as particles and interfere with detection of offending particles. A tool that holds multiple containers for consistent presentation can be useful when performing inspection. Holding many containers by hand at once should be avoided, as it is difficult to obtain a complete view of all container surfaces and contents. Container motion or rotation is also helpful for identifying small container defects such as cracks or chips.

Magnification: Some inspection processes use a large magnifier to increase image size and thus increase the probability of detecting and rejecting containers with defects near the threshold of detection. Although magnification can be useful for critical examination of a portion of the container, it does not often lead to increased overall detection rates for defects of interest. This may be due, in part, to the added eye strain that often results from use of magnification. As such, it is not recommended as part of the reference inspection method described in ⟨790⟩ or in other global pharmacopeias (*55,57*). Although not recommended for use during routine inspections, magnification can be helpful for critical examination of a small number of units, as may be needed during an investigation.

INSPECTOR FATIGUE AND ERGONOMIC CONSIDERATIONS

Inspecting for extended periods of time can cause inspector fatigue and a decrease in inspection performance. Based on industry experience (*43*), it is recommended that inspectors be given a break from performing inspection at least every hour. This break should allow time to rest the eyes and mind, and may be achieved with a short rest (e.g., 5 min) or a longer meal break. This need for regular breaks may also be met through rotation to a noninspection function, such as material handling or documentation.

Inspection stations should be designed and operated in a manner that minimizes the inspector's risk of repetitive-motion injury. Adjustable chairs and careful positioning of light sources as well as incoming and inspected product can reduce the risk of such injury. These adjustments can also reduce inspector fatigue and discomfort, both of which can be distracting and thus can decrease performance.

The inspection room environment should also be considered. Temperature and humidity should be controlled for inspector comfort. Reduced ambient lighting is recommended to focus the inspection process and to reduce distraction from extraneous reflections. Special care should be given to inspection rooms with exterior windows that allow daylight into the room and thus changing ambient lighting throughout the day and with changing seasons.

6.2 Semi-Automated Visual Inspection

Semi-automated visual inspection combines automated material handling of the containers to be inspected with human vision and judgment to make the decision to accept or reject. These systems often use a conveyor equipped with rollers to transport the containers in front of the inspector inside an inspection booth or station. For inspection of liquids, the booth can be equipped with a high-speed spin station to set particles in motion. The rollers are also used to slowly rotate the containers in front of the inspector as they traverse the inspection zone. These systems offer a means to control the presentation of the vials and can offer additional lighting options, such as Tyndall lighting, which may enhance the appearance of some defects such as cracks or small particles. Mirrors may also be used to provide a clear view of the top and bottom of each container. Rejected units may be removed from the rollers by hand, and some systems are equipped with a remote rejection system that can be triggered by the inspector. Care should be taken in the qualification and operation of these systems to ensure full rotation of vials in the inspection zone; this allows examination of all surfaces. In addition, studies should be conducted to ensure the detection of heavy particles, which may not be lifted from the bottom of the container, and to ensure that the rate of inspection produces an acceptable detection rate for defects of interest.

With semi-automated visual inspection, performance is similar to that with MVI. Some increase in throughput may be achieved because the inspector spends all of the available time viewing the containers, rather than splitting the time between inspection and material handling.

CRITICAL PROCESS PARAMETERS FROM SEMI-AUTOMATED INSPECTION

Light intensity must be controlled, as with MVI. The rate of inspection is controlled by the speed of the roller/conveyor or some equipment that allows the inspector to call for a group of containers each time. Spin speed for liquid products and rotation rate for all containers should be established during validation/qualification and maintained within the validated range for routine inspection. The background color is controlled by the color of the rollers selected and the color of the background seen through the spaces between the rollers. Qualification of inspectors and validation of the inspection equipment should be based on

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comparison with the compendial manual inspection process with an expectation that alternative methods such as semi-automated inspection demonstrate equivalent or better performance.

6.3 Automated Visual Inspection

Automated visual inspection (AVI) combines automated material handling of the containers with electronic sensing of product appearance. Containers that do not meet pre-programmed acceptance criteria are automatically rejected by the machine. Early machines performed inspection for particles and fill level, but manual or semi-automated inspection was required for the container and closure system. Newer models have the capability to inspect all attributes of the containers, along with the contents. As with MVI, machines often spin the containers to set particles in motion and make them easier to detect. Multiple cameras are used to image various regions on the container in great detail. Each camera is coupled with unique lighting to highlight specific defects in the region of interest. Light-field and dark-field lighting techniques offer the same benefits as white and black backgrounds as discussed above, offering contrast for a full range of light- and dark-colored defects. A defect found by any camera is tracked through the machine to allow accurate ejection by the reject system. These machines also offer detailed reporting of defects observed in a specific production lot.

AVI offers advantages in the areas of throughput and consistency, compared with MVI (*4*). AVI may also offer enhanced sensitivity for some defects, compared with MVI, but may suffer from higher false rejection rates due to the inability to tolerate normal variation in containers or product. This is especially true for molded glass containers and flexible bags.

Validation of the automated inspection equipment should be based on comparison with the compendial manual inspection process with an expectation that alternative inspection methods demonstrate equivalent or better performance. Significant effort is required to program these systems and to test their performance against a range of known defects, as well as acceptable containers.

LIGHT-OBSCURATION METHODS

Some systems use an optical sensor to detect the shadow of particles in solution products. This technique has been used successfully for detection of both subvisible and visible particles. For the detection of visible particles, it requires particles to be in motion, typically using a high-speed spin and rapid braking of the container to achieve this motion. Spin conditions must be optimized to provide sensitivity for heavier particles while minimizing false rejections due to bubbles. Some biological products experience shear-induced agglomeration, so care should be taken with regard to agitation of these products.

LO methods are optimized for sensitivity to moving particles, and can thus be made less sensitive to minor container imperfections. These methods can be used with both tubing and molded containers. Results are generally robust in detecting single particles that are 100 µm in diameter and larger, though detection of smaller particles at or below the visible threshold is improved when multiple particles are present.

These systems can also detect fill height by detecting the shadow of the solution meniscus. Generally, this process is not sensitive enough to ensure compliance with dose or fill-weight specifications, but it can provide a secondary check of gross fill. Sensitivity is a function of the container shape, with greater sensitivity achieved in small-diameter containers.

IMAGING METHODS

Continuing advances in camera technology now allow the rapid capture of high-resolution images for inspection. When coupled with high-speed processors that have ever-increasing computational capability, a powerful inspection tool can result. Images are divided into inspection windows, and an array of tools such as image subtraction, pixel counting, intensity analysis, and others are used to assess the images against programmed quality attributes.

Imaging systems can detect particles and fill level, as well as other container and closure attributes. Inspection in this manner can provide comprehensive inspection of visual attributes. These systems can offer high sensitivity, but may also have high false-rejection rates if container and product attributes are not tightly controlled.

X-ray imaging has also been explored as a means to detect particles within freeze-dried cakes, powders, or suspensions (*59*).

These technologies may be used alone or in combination with other inspection methods to provide a comprehensive assessment of product quality before labeling and packaging.

7. QUALIFICATION AND VALIDATION OF INSPECTION PROCESSES

7.1 Standards

The use of standards for visual inspection has been described by Melchore and Berdovich (*60*). Development of inspection standards begins with description of the defect types that will be represented in the test set(s). This information typically comes from the manufacturing area, where naturally occurring defective units can be identified from rejected product. The defects are categorized as critical, major, or minor. These defects must be further characterized to allow for (a) selection from naturally occurring particulate and physical or cosmetic production rejects removed from product lots, and/or (b) re-creation of equivalent defect types in a controlled laboratory environment. Characterization information on defects should include, where appropriate, the range of sizes typically observed and the specific location on the container. If feasible, a photograph of the defect should be included. All information that could support consistent re-creation of the defect standards should be included in the characterization description.

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7.2 Preparing Defect Standards

Visual inspection standards may be identified from known production rejects, or may be created manually with characterized particulate material. A single particle per seeded container should be used when determining detection thresholds. The focus should be to demonstrate single particle detection sensitivity especially at the edge of the reject and grey zones. The use of multiple particles in a container is not recommended in order to avoid skewing the data by increasing the PoD.

7.3 Particle Types

The primary packaging materials that directly contact the product and the potential environmental contaminants can be divided into specific particle groups such as glass, stainless steel, elastomeric closure, plastic, and fibers (synthetic or natural). Naturally occurring visible particles from production rejects containing these materials may be used for training and to assess inspection performance. Physically prepared particles can be sieved initially to target a specific size, and then the individual particles are measured using optical microscopy prior to seeding into a prepared container. The use of characterized production defects or physically prepared standards is preferred over standard spherical particles for inspector training and qualification, as well as machine validation as they better represent actual inspection challenges and performance.

Spherical standard particles may be utilized as surrogates for naturally occurring particulates; however, these are best used for routine machine calibration rather than validation or inspector qualification, as they do not move or look like actual production defects.

7.4 Rejection Probability Determination

Once a well-defined defect standard is available, it is assigned a detection frequency or PoD by conducting a documented, manual human inspection qualification that is accomplished by repeated manual inspection. This repeated inspection is the basis for qualifying the defect standard. This approach has been described by Knapp and Kushner (*52,53*). The Knapp methodology recognizes that the detection of particles is probabilistic, and repeated inspections with strict controls on lighting and inspection pacing/sequencing generate the statistical confidence to assign a reject probability to each standard unit. A manual, visual inspection PoD of ±0.7 or 70%, is required to assign the container to the reject zone for subsequent calculation of the reject zone efficiency (RZE). Secure probabilistic data for particulate standards can be achieved with 30–50 inspections of each container. This is best achieved with multiple inspectors. Inspection reject probability is calculated for the defect as follows:

$$\text{PoD} = (\text{Number of times rejected})/(\text{Number of times inspected})$$

7.5 Test Sets

These qualified defect standard units are then assembled into test sets, which may be used to specifically challenge the particle detection technique of human inspectors, used as part of a defect test set (including container–closure defects) for human qualification, or for comparison during automated equipment qualification and validation. When possible, the test set should be prepared with duplicate product units per particle type and size to ensure that backup units are available in the event that a standard container is broken or the particle is trapped or lost within the container. When using test sets, it is a good practice to verify the presence of particles before and after use, as particles may become lodged between the container and the closure. When a freely moving particle cannot be verified in liquid formulations, the unit should not be used and the data should be excluded from subsequent calculations. When this happens, it may be possible to free the particle with the use of an ultrasonic bath. If this is not possible, the unit should be replaced. For dry solid formulations, such as powders or lyophilized cakes, the appearance of the standards may degrade with repeated handling, requiring frequent replacement and adding to the difficulty in performing these types of studies. The number of defective units in each test set should be limited to approximately 10% to prevent rejection bias (*57*). The accept containers will be identified as having a predetermined manual, visual inspection PoD of <0.3 or 30%. The accept zone containers are used as blank units or may be used to study the false reject rate of the inspection method. Any particle standards found to fall within the acceptable "grey zone", indicating a manual inspection rejection probability ≥30% and <70%, may be included as an "acceptable unit" in a test set, if desired. The true utility of the grey zone units is to study the units from ≥50% and <70% PoD and demonstrate the manual detection frequency of subvisible particles just below the reject zone threshold. This data can be used for illustrating the subtle differences between the manual inspection and alternate inspection methods (semi-automatic or fully automatic) during comparison studies. The human efficiency in detecting grey zone containers should be used for information only and formal acceptance criteria is not typically applied to grey zone containers (*61*).

It is important to prepare a written procedure for the creation and maintenance of standards. This procedure should define the qualification criteria, appropriate storage conditions, periodic examination and requalification, expiration, and sample custody during use. Test sets should be approved by the quality unit. The container in which the specific particle set is stored must be clearly labeled with the test set identification information.

7.6 Types of Test Sets

The particle detection threshold can be determined for a specific inspection method and product/package combination. It is a standard curve of detection probabilities at various particle types and sizes in an approximate range of 100–500 µm (with recommended increments of 100 µm). Fibers are typically observed in sizes >500 µm. The typical size range of particles used in threshold studies incorporates a variety of particle types and densities that are typically found in the manufacturing environment.

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Threshold studies are conducted to determine the sensitivity of manual inspection methods, using a range of particle sizes, in a blinded study that yields the particle-size detection capabilities of a defined group or of an individual inspector. The threshold studies indicate that the method of inspection is valid and appropriate. For example, for clear solutions in 10-mL tubing glass vials, past threshold studies indicate that particles within the range of 150–250 µm (500–2000 µm for fibers) can be detected with a PoD of 70% or greater. Results can differ due to differences in product formulation as well as container type and size. Threshold studies are also useful as an assessment tool when evaluating or qualifying visual inspection staff on a specific method with fixed testing parameters. Detection threshold studies are typically the first step in evaluating the performance of any new inspection method.

Depending on product and/or presentation, rejects in the test set should represent all defects anticipated for a given container type or product family. For particles, use a bracketed range of types (densities) and sizes from near the lower limit of the visible range (100 µm) to the largest routinely observed in the pool of rejects. For an individual manual test set, it is important that all containers and closures are of the same type, and the samples are blinded. UV ink (invisible to the inspectors) may be used to mark all containers. Alternatively, bar codes or other coded labels may be used. Manual test sets can be used initially to qualify, or periodically to re-qualify, human inspectors. These test sets may also be used for direct comparison to semi-automated or automated inspection methods. If significantly different formulations (e.g., clear solution, suspension, lyophilized) or packages (e.g., clear vials, amber vials, ampoules, syringes) are produced at the same facility, separate test sets should be prepared to represent each unique combination. A bracketing approach may be used with regard to different container sizes.

7.7 Training and Qualification of Human Inspectors

Before training, potential inspectors should be tested for visual acuity (*62*) and color perception. Near-vision performance should be the equivalent of 20/20 with no impairment of color vision. Both the Snellen and Jaeger charts are useful for verifying visual acuity; they test far and near vision, respectively. The use of corrective lenses to achieve the desired visual acuity is permitted. Training should include a phased approach with a specified number of training hours expected for each segment. Initially, train the potential inspectors with defect photographs or a video library and clear, written descriptions. Utilize subject matter experts to mentor and provide hands-on training with defect standards for the specified method. Reinforce mental or silent counting and follow the paced sequence to achieve consistent inspection timing. Stress the importance of strict adherence to the inspection process (procedure, sequence, and timing). Inspector fatigue may be addressed in the qualification process by testing under worst case conditions (e.g., at the end of a typical inspection shift). Train all inspectors (QC, QA, and production) with common procedures used for 100% inspections and AQL inspections. All inspection practices should be standardized and consistently executed across all inspection groups.

Qualification should be performed for each product type and package that the inspector will encounter. A bracketed or matrix approach can be used to simplify qualification of products with similar physical or visual characteristics such as container type and size, formulation type, product viscosity, color, and others. It is common to initially train and qualify personnel on clear solutions in clear containers (if produced at the facility) and then expand their expertise to inspection of more difficult formulations or presentations.

7.8 Inspector Qualification Requirements

The qualification of all inspection personnel utilizes a manual test set to be inspected under normal operating conditions and inspection critical parameters, including inspection timing and sequence, physical environment, and inspection duration. Three successful inspections of the test set are recommended to demonstrate consistent performance for initial qualification of new inspectors. Acceptance criteria for each defect class should be based on the PoD (or RZE) observed during test set qualification. A limit is also needed for false rejection, with a recommended target of <5% falsely rejected good units.

7.9 Requalification

Inspectors should be requalified at least annually. Requalification includes a test of visual acuity and testing with at least one product/test set configuration. A single successful inspection of the test set is sufficient for requalification. Requalification may also be necessary in the event that poor performance is observed during routine inspection or if the inspector has been away from the inspection operation for an extended period of time (e.g., 3 months).

If an inspector fails the requalification test, a retraining process should be initiated to identify the root cause and allow the inspector to receive additional instruction. After this process has been completed, the inspector may attempt to meet the acceptance criteria one additional time. If the inspector fails, he or she may attempt to qualify again after a specified time period.

8. PRODUCTS IN DISTRIBUTION

Chapter ⟨790⟩ states, "If it becomes necessary to evaluate product that has been shipped to customers (e.g., because of a complaint or regulatory concern), sample and inspect 20 units. If no particles are observed in the sample, the batch is considered essentially free of visible particulates. If available, additional units may be inspected to gain further information on the risk of particulates in the batch."

For products in distribution, questions regarding batch quality will occasionally arise from customer complaints, observations in the field, customer use questions and from the use of nonstandard (sensitive) conditions of inspection. As discussed in this chapter, the detection process is probabilistic and the likelihood of detection is a cumulative function of the particle's visible attributes, drug product and container characteristics, and the inspection method used. In an appropriately qualified manufacturing process, the batch is presumed to have been prepared according to robust processes and all containers with package defects and visible particles (nonconforming units) removed prior to labeling. In that regard, the evaluation outlined

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in general chapter *Visible Particulates in Injections* ⟨790⟩, *Inspection Procedure, Sampling at Batch Release (After 100% Manufacturing Inspection)*, and *Product in Distribution* is only permissible if both sampling at batch release and a 100% manufacturing inspection have been successfully completed.

The particle detection threshold should be determined for a specific inspection method and product/package combination incorporating a variety of particle types and densities that are typically found in the manufacturing environment. For example, the detection threshold for routine, reliable detection (≥70% probability) of a single spherical particle in a clear solution contained in a 10-mL vial utilizing diffuse illumination between 2,000 and 3,000 lux is often near 150 µm in diameter (*4*). Units returned from distribution may be false positive, may contain particles larger than the acceptance threshold that were missed, may contain particle(s) in the "grey zone," e.g., less than the detection threshold, or may have suffered a physicochemical change that resulted in a visible change. Ideally there were no visible particles in the containers released to market; however, there is always a low probability that this may occur.

Upon receipt, suspect containers should be subjected to the same inspection conditions and methodology used in the release inspection. Particle(s) verified in the returned or re-evaluated supply must be carefully characterized by an analytical forensic process to determine their source and likely cause. Single particles of typical product-contact materials are unlikely to present a concern. Multiple particles, large particle sizes, and any particles indicative of physical or chemical change are significant events and should be subject to further investigation. Rare instances of particulate material falling into the "grey zone" should be expected given the probabilistic nature of the inspection process and should not routinely trigger further evaluation of retention samples. Chapter ⟨790⟩ provides that zero particles found in the sampling and inspection of 20 units signifies that the batch is "essentially free of visible particulates." If multiple suspect containers from the same batch are detected, additional units should be inspected and an appropriate rationale provided to support the batch's conformance to the registered specifications.

Overall batch quality using internal systems to control particulate matter and the means to investigate these occurrences is key to the life-cycle approach for modern pharmaceutical production. Evaluation of retention and stability samples provides insight to batch quality, as do the field-use effects for any medication. While the presence of particles or product or container defects discovered in retained or returned product do not necessarily incriminate the quality of the batch, careful investigation should be conducted to exclude systemic risks.

9. CONCLUSIONS AND RECOMMENDATIONS

Visual inspection for particles and other visible defects continues to be an important part of the manufacturing process for injections. Chapter ⟨790⟩ provides a useful reference method and acceptance criteria for visible particulates in injections. Successful execution of visual inspection requires an understanding of the inspection process and careful control of inspection conditions. Inspectors must be trained to ensure consistent, high-quality performance. Alternative inspection methods, either semi-automated or fully automated, may be used in place of manual inspection methods. Where machine methods are used, the equipment must be validated to demonstrate equivalent or better performance when compared to manual inspection. The use of test sets that contain standard defects is an important element in inspector training and qualification as well as machine validation. Good product development will lead to a stable product with a lower risk of particle formation. Identification of the type or types of particles found during product development and routine manufacturing is an important aid in source identification and reduction. Inspection results should be trended to further aid in continuous process improvement with the ultimate goal of defect prevention.

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⟨1821⟩ RADIOACTIVITY—THEORY AND PRACTICE

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1. INTRODUCTION

Radioactive drugs and devices require specialized techniques in their production, testing, handling, dispensing, and administration to ensure optimal effectiveness and maintain safety for workers, patients, and the public. All operations involving these articles should be carried out by or under the supervision of personnel who have been appropriately trained in the handling of radioactive materials.

The facilities for the production, storage, and use of radioactive drugs and devices are generally subject to licensing by the U.S. Nuclear Regulatory Commission, an appropriate state agency, or similar governmental agencies outside of the United States. Most radioactive drugs and devices, although not identified as hazardous drugs, are classified as hazardous materials and are therefore subject to other regulations relating to transportation, environmental release, and workplace safety.

The purpose of this chapter is to provide information regarding radioactivity—including definitions, types of decay, and general considerations relating to radioactive decay, counting, radionuclide production, purity, and labeling—as well as instrumentation for detection and measurement of radioactive emissions.

Specific information on standards for radionuclide identification and assay, including instrument qualification, performance checks, identification of radionuclides and radionuclidic impurities, and assay of radionuclides are provided in *Radioactivity* ⟨821⟩.

2. TYPES OF DECAY

"Radioactive decay" is the process by which an unstable nuclide transitions to a lower energy configuration. Depending on the particular starting radionuclide, the result of the transition may be either a stable nuclide or a different radionuclide. Typically, these transitions are accompanied by the emission of radiation from the nucleus, which is broadly classified as either particulate or nonparticulate. Some radionuclides emit multiple types of radiation in this process, whereas others emit only a single type. The main types of particulate radiation commonly seen in nuclear medicine are alpha, beta, and positron. Nonparticulate types of radiation include gamma rays and X-rays. Nuclear medicine imaging is accomplished through detection and localization of nonparticulate radiation, whereas therapeutic effects arise from the energy deposited in the target organ by particulate radiation.

2.1 Alpha Decay

"Alpha decay" is radioactive decay with the emission of alpha particles, or helium nuclei, and is generally limited to elements with an atomic number >83. In some cases, beta particles and gamma rays may also be emitted during alpha decay. An example of a radionuclide that decays by alpha decay is:

Radium-226 → Radon-222

2.2 Beta Decay

"Beta decay" is radioactive decay with the emission of an electron. This type of decay typically occurs in neutron-excessive radionuclides wherein a neutron is transformed into a proton. In some cases, the emission of a positively charged electron, or positron, may occur. This type of decay typically occurs in neutron-deficient radionuclides with a lower atomic number wherein a proton is transformed into a neutron. In some cases, gamma rays may also be emitted during beta decay. An example of beta decay through emission of an electron is:

Iodine-131 → Xenon-131

An example of beta decay through emission of a positron (β^+) is:

Fluorine-18 → Oxygen-18

Because a positron is an anti-electron, when it interacts with an electron, the two particles annihilate, and their combined mass is transformed into energy in the form of two 511 kiloelectron volt (keV) gamma rays. These gamma rays are produced simultaneously and travel away from the point of interaction in nearly opposite directions. These two characteristics form the basis for positron emission tomography (PET) imaging techniques.

2.3 Electron Capture Decay

"Electron capture" is radioactive decay that involves nuclear capture of an inner orbital electron, nuclear transformation of a proton into a neutron, and emission of one or more gamma rays. Electron capture generally occurs in higher atomic number radionuclides that are neutron deficient. An example of a radionuclide that decays by electron capture decay is:

Iodine-123 → Tellurium-123

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2.4 Isomeric Transition

An "isomeric transition" is radioactive decay that involves a transition between nuclear isomers with the emission of one or more gamma rays. In contrast to other types of decay, the number of protons and neutrons remains the same in an isomeric transition. Isomeric transition generally occurs in radionuclides that are metastable. An example of a radionuclide that decays by isomeric transition is:

Technetium-99m → Technetium-99

3. GENERAL CONSIDERATIONS

3.1 Radioactivity

Radioactive decay is a first-order process (i.e., a fraction of atoms decay per unit time). The rate of decay for each radionuclide is a unique and constant value, which gives rise to the term "decay constant".

Each radionuclide's rate of decay is a unique and constant value (its decay constant) and can be described by the following equation:

$$A = \lambda N$$

A = amount of radioactivity in a source at a given time
λ = rate of decay of the radionuclide
N = number of radioactive atoms

The traditional unit for radioactivity is the curie (Ci), which is equal to 3.7×10^{10} atoms undergoing radioactive decay, or disintegrations per second (dps). Commonly used prefixes associated with the Ci include the millicurie (mCi) and the microcurie (μCi). The SI unit for radioactivity is the becquerel (Bq), which is equal to 1 dps. Commonly, prefixes associated with the Bq include the megabecquerel (MBq) and the gigabecquerel (GBq). Hence, 1 Ci = 37 GBq.

3.2 Fundamental Decay Law

The decay of a radioactive source is described by the equation:

$$N_t = N_0 e^{-\lambda t}$$

N_t = number of radioactive atoms remaining at elapsed time t
t = time elapsed (time unit, such as seconds, minutes, or hours)
N_0 = number of radioactive atoms when $t = 0$
λ = decay constant of the specific radionuclide

The above equation can be rewritten in terms of radioactivity:

$$A_t = A_0 e^{-\lambda t}$$

A_t = amount of radioactivity at elapsed time (t)
t = time elapsed (time unit, such as seconds, minutes, or hours)
A_0 = amount of radioactivity when $t = 0$
λ = decay constant of the specific radionuclide

"Decay tables" that provide radionuclide-specific decay factors (i.e., fraction remaining) calculated from $A_0 e^{-\lambda t}$ at various elapsed times (t) are commonly available.

The half-life is defined as the time interval required for a quantity of radioactivity to decay to one-half of its initial value and is related to the decay constant λ by the equation:

$$T_{1/2} = 0.69315/\lambda$$

$T_{1/2}$ = half-life of the radionuclide
λ = decay constant of the specific radionuclide

The activity of a pure radioactive substance as a function of time can be obtained from the exponential equation, from decay tables, or by graphical means based on the half-life (*Figure 1*).

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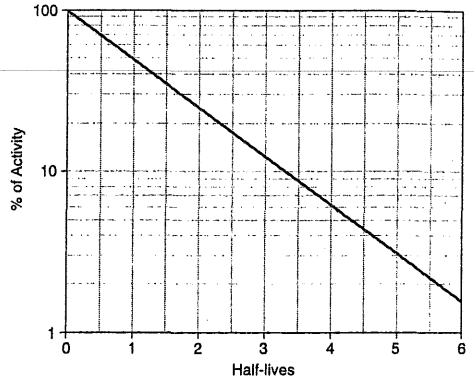

Figure 1. Normalized decay chart.

3.3 Counting Efficiency

The validity of any radionuclide measurement is dependent upon the reproducibility of the relationship between the source, the detector, and its surroundings. Appropriate allowances should be made for source configuration. Measurements of radioactivity require calculation of a calibration factor, or efficiency, and are dependent on the type of detector, the container in which the radioactivity is placed, and the source–detector geometry. The basic efficiency equation for detectors designed to count discrete decay events over a period of time is given as:

$$\text{disintegrations/s} = (\text{counts/s})/\varepsilon$$

ε = efficiency or calibration factor

Because 1 dps is defined as 1 Bq, the above equation gives radioactivity in units of Bq. Through the application of the appropriate unit conversion, Bq can be transformed into μCi or any other unit of radioactivity. Detectors should be calibrated with a source of known radioactivity and in a fixed geometry.

3.4 Background

Cosmic rays, radioactivity present in the detector or shielding materials, and radiation from improperly shielded sources contribute to the background radiation. All radioactivity measurements should be corrected by subtracting the background count rate from the gross count rate in the test sample.

3.5 Statistics of Counting

Modern radiation detection systems often incorporate statistical analysis into their software. The user should understand the use and limitations of these programs to ensure accurate results.

Because the process of radioactive decay is a random phenomenon, the events being counted form a random sequence in time. Therefore, counting for any finite time can yield only an estimate of the true counting rate. The precision of this estimate, being subject to statistical fluctuations, is dependent upon the number of counts accumulated in a given measurement and can be expressed as:

$$\sigma = \sqrt{n}$$

σ = standard deviation
n = number of counts accumulated in a given measurement

The probability of a single measurement falling within $\pm 100/\sqrt{n}\%$ of the mean of a great many measurements is 0.68, which means that if each count were to lie within $\pm 100/\sqrt{n}\%$ of the mean for approximately two-thirds of the observations, then approximately one-third of the observations would lie outside of this interval.

Because of the statistical nature of radioactive decay, repeated counting of an undisturbed source will yield count-rate values in accordance with the frequency of a normal distribution. Any deviations in these values from the normal distribution conform to the chi-square (χ^2) statistical test. For this reason, the χ^2 test is frequently applied to determine the performance and correct operation of a counting assembly. The term "figure of merit" of a radioactive counting instrument is expressed as:

$$\text{Figure of merit} = \varepsilon^2/B$$

ε = counter efficiency
B = background count rate (cps)

In the selection of instruments and conditions for assay of radioactive sources, the figure of merit should be maximized.

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3.6 Minimum Detectable Activity

In situations where only very small quantities of radioactivity are to be measured, the lower limit of the ability of the instrument to detect that particular radionuclide should be known. The "minimum sensitivity", also referred to as "limit of detection", is defined as the net count rate above background that must be exceeded before a sample is deemed to contain detectable radioactivity with a specified level of confidence. The minimum sensitivity is generally considered to be 3 standard deviations above the mean background count rate and is calculated as:

$$\text{Minimum sensitivity} = (3 \times \sqrt{B})/t$$

B = background count rate (cps)
t = count time

The minimum detectable activity is defined as the smallest quantity of radioactivity that can be measured under the specific conditions of minimum sensitivity and counting efficiency of the instrument. It is calculated as:

$$\text{Minimum detectable activity} = (\text{Minimum sensitivity})/(\varepsilon \times F)$$

ε = counting efficiency
F = unit conversion factor

For example, if minimum sensitivity is in units of dpm and minimum detectable activity is desired to be in units of Bq, then F = 60 dpm/Bq. If minimum sensitivity is in units of cpm and minimum detectable activity is desired to be in units of µCi, then F = 2.2×10^6 cpm/µCi.

3.7 Limit of Quantification

The limit of quantification is the smallest quantity of radioactivity that can be quantitatively determined with suitable precision and accuracy. The limit of quantification is used particularly for the determination of impurities and degradation products. In practical terms, the limit of quantification is usually considered to be 10 standard deviations above the mean background count rate.

3.8 Counting Losses

The minimum time interval that is required for the counter to resolve two consecutive signal pulses is known as the dead time. The dead time is typically on the order of microseconds for proportional and scintillation counters and to hundreds of microseconds for Geiger counters. Nuclear events occurring within the dead time of the counter will not be registered. The corrected count rate, R, can be calculated using the formula:

$$R = r/(1 - r \times \tau)$$

r = observed count rate
τ = dead time

The correction formula assumes a nonextendable dead time. The observed count rate, r, refers to the gross sample count rate and is not to be corrected for background before use in this equation. For general validity, the value of $r \times \tau$ should not exceed 0.1. Most modern counting systems have the ability to account for dead time, but dead time may still be a factor to consider in some circumstances.

3.9 Linearity and Range

When a radiation detection instrument is used in a quantitative measurement, the instrument should be suitable for the type(s) of radiation to be measured, and the instrument response should be linear over the range of measurements or a correction factor should be applied. Normally, a minimum of five different quantities of radioactivity are used to establish linearity. These quantities should bracket the range of radioactivity levels that are routinely measured in a particular application.

3.10 Calibration Standards

All radioactivity assays should be performed using measurement systems that have been calibrated with appropriately certified radioactivity standards. Such calibration standards may be purchased either directly from an appropriate National Metrology Institute (NMI) or from other sources that have established traceability to the NMI, through participation in a program of inter-comparative measurements. Where such calibration standards are unavailable, nuclear decay data required for calibration can be obtained from the Evaluated Nuclear Structure Data File maintained at the Brookhaven National Laboratory.[1]

[1] http://www.nndc.bnl.gov/ensdf.

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3.11 Production of Radionuclides

The various radionuclides found in nature generally have undesirable properties for nuclear medicine applications, including very long half-lives (e.g., thousands or millions of years), decay with emissions of alpha or beta particles, and low isotopic purity because of the presence of other isotopes of that element. Because of these properties, naturally occurring radionuclides are rarely used for radiopharmaceuticals, except for some alpha-emitting members of the actinide decay series, which are used for certain therapeutic radiopharmaceuticals (e.g., radium-223).

There are four common production routes for artificially produced radionuclides: fission, neutron activation, charged-particle-induced reactions (cyclotron), and radionuclide generators.

"Fission byproducts" refer to radionuclides that are obtained as byproducts of the fission of uranium (uranium-235). These radionuclides, whether directly produced fission fragments or subsequent members in a decay chain originating from a fission fragment, can be chemically separated into individual forms from the mixture of fission products. Desirable properties of fission byproducts include high isotopic purity and moderate cost. Undesirable properties may include beta-particle emissions, low specific activity of certain radionuclides, and the limited selection of radionuclides produced. Examples of fission byproducts used in nuclear medicine applications include iodine-131 and xenon-133. Currently, the most widely used fission byproduct is molybdenum-99, which is used in technetium-99m generators.

"Neutron activation" refers to the production of radionuclides in a nuclear reactor by bombarding target atoms with thermal neutrons. Nuclear transformations, induced by neutron capture, result in isotopes possessing one additional neutron, and thus an atomic mass increased by one. The excess energy of the newly formed isotope is emitted as a gamma ray. These reactions are often termed (n,γ) reactions. Desirable properties of neutron-activated radionuclides include a wide variety of isotopes that can be produced and the moderate cost to produce them. Undesirable properties may include decay with beta-particle emissions and relatively low isotopic purity (i.e., unreacted stable target atoms are mixed with their radioisotope products). Because of their beta emissions, however, several of these radionuclides have been used in therapeutic radiopharmaceuticals. Examples of neutron-activated radionuclides used in therapeutic radiopharmaceuticals include strontium-89, yttrium-90, iodine-131, samarium-153, and lutetium-177.

Cyclotron production of radionuclides occurs by bombarding stable atoms with charged particles (e.g., protons or deuterons) that have been accelerated in the cyclotron's oscillating electromagnetic field. Nuclear transformations induced by particle capture usually result in a radioisotope of a different element with the emission of one or more neutrons or protons. For example, if a proton is captured and a neutron is emitted, the reaction is often termed a (p,n) reaction. Desirable properties of cyclotron-produced radionuclides include the wide variety of isotopes that can be produced, the availability of alternate production schemes, radionuclide decay by electron capture or positron decay rather than by beta decay, and high isotopic purity. Undesirable properties may include contaminating radioisotopes from side reactions and the relatively high cost of radionuclide product. Examples of cyclotron-produced radionuclides used in nuclear medicine applications include carbon-11, fluorine-18, gallium-67, indium-111, iodine-123, and thallium-201.

Generators refer to a special method of radionuclide production whereby a short-lived radionuclide results or is generated from the decay of a longer-lived radionuclide. The longer-lived parent radionuclide is generally bound to a column, and the short-lived radionuclide daughter product is then extracted (eluted) from the column. After elution, subsequent decay of the long-lived parent radionuclide generates more of the short-lived radionuclide daughter product, which can then be extracted. A generator provides a specific radionuclide in sequential elutions over a prolonged period of time. Desirable properties of generator-produced radionuclides include ready availability, portability, low-to-moderate cost, variety of radionuclides and type of decay, and relatively high isotopic purity. Undesirable properties may include the limited number of parent-daughter pairs and the potential for generator breakthrough of the parent radionuclide in the eluate. Examples of generator-produced radionuclides used in nuclear medicine applications include technetium-99m (daughter of molybdenum-99), rubidium-82 (daughter of strontium-82), and gallium-68 (daughter of germanium-68). The characteristics of all four production methods are summarized in *Table 1*.

Table 1. Production Methods of Radionuclides

Production Method	Nuclear Reactor (fission byproduct)	Nuclear Reactor (neutron activation)	Cyclotron	Radionuclide Generator
Bombarding particle	Neutron	Neutron	Proton, deuteron, triton, alpha	Production by decay of parent
Product	Neutron excess	Neutron excess	Neutron poor	Neutron poor or excess
Typical decay pathway	β^-	β^-	Positron emission, electron capture	Several modes
Typically carrier free	Yes	No	Yes	Yes
High specific activity	Yes	No	Yes	Yes
Relative cost	Moderate	Moderate	High	Low to moderate
Radionuclides for nuclear medicine applications	Molybdenum-99, iodine-131, xenon-133	Iodine-131, strontium-89 yttrium-90, samarium-153, lutetium-177	Thallium-201, iodine-123, gallium-67, indium-111, fluorine-18, carbon-11	Technetium-99m, krypton-81m, gallium-68, rubidium-82

Other methods of radionuclide production have been developed but currently are not used to produce radionuclides used in radiopharmaceuticals.

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3.12 Carrier

The total mass of radioactive atoms or molecules in a radiopharmaceutical is directly proportional to the amount of radioactivity and is usually too small to be measured by ordinary chemical or physical methods. For example, 37 MBq (1 mCi) of iodine-131 has a mass of 8×10^{-9} g. Because such small quantities behave in an anomalous manner, such as nonspecific adsorption to container walls, a carrier may be added during processing to permit ready handling. Amounts of the carrier, however, should be sufficiently small so that undesirable physiological, pharmacological, or toxicological effects are not produced. Also, because the carrier is chemically identical to the radionuclide, the amount of carrier should be limited to avoid competitive interference with the desired chemical reactions and overall radiochemical yield.

The term "carrier free" refers only to radioactive preparations in which other isotopes of the radionuclide are absent (i.e., free from the presence of carrier). In practice, a true carrier-free state may be difficult or impossible to achieve because of the ubiquity of certain elements or molecules. Hence, the term "no carrier added" may more appropriately describe a preparation that may contain a trivial amount of carrier but for which additional carrier has not been purposefully added. Radionuclides produced by neutron activation reactions generally contain substantial amounts of nonradioactive isotope remaining from unreacted target material and thus cannot be considered carrier free. However, there are select cases in which this is not the case, such as (n,p) reactions.

The radioactivity per unit volume of a medium or vehicle containing a radionuclide is referred to as the "radioactivity concentration", "specific concentration", or "strength" and is expressed in units such as Bq/mL or Ci/mL. The radioactivity of a radionuclide per unit mass is referred to as "specific activity" and is expressed in units such as Bq/g, Ci/g, or Bq/mol. The maximum specific activity of a radioactive preparation exists when it is in a carrier-free state; the addition of a carrier results in lowered specific activity.

3.13 Radiochemical Identity and Purity

"Radiochemical identity" may be defined as the molecular structure of the compound that contains the radionuclide. Because it is nearly impossible to analyze the structure of radiolabeled compounds with the traditional tools used for organic structure determination, the radiochemical identity of a radiopharmaceutical is often determined indirectly. This process begins with the preparation and characterization of a nonradioactive analog, which is commonly referred to as the "cold compound". The radiolabeled compound is often chromatographically analyzed simultaneously with the cold compound. The identical response of the two compounds demonstrates the structural identity of the radiolabeled compound.

The radiochemical purity of a radiopharmaceutical preparation refers to the fraction of the stated radionuclide present in the stated chemical form. Radiochemical purity is important for radiopharmaceuticals because radiochemical impurities may affect the biodistribution and interfere with image interpretation (diagnostic accuracy). In addition, radiochemical impurities may alter radiation absorbed doses to various organs. When using therapeutic radiopharmaceuticals, radiochemical purity is very important because altered biodistribution associated with radiochemical impurities may result in insufficient irradiation of the target tissue (suboptimal treatment response) or excessive irradiation of other tissues (undesired radiation damage).

Radiochemical impurities in radiopharmaceuticals may result from byproducts of the preparative method or from decomposition. Radiation causes decomposition of water, a main component of aqueous radiopharmaceutical preparations, leading to the production of reactive hydrogen atoms and hydroxyl radicals, hydrated electrons, hydrogen, hydrogen ions, and hydrogen peroxide. Hydrogen peroxide is formed in the presence of oxygen radicals, originating from the radiolytic decomposition of dissolved oxygen. Many radiopharmaceuticals show improved stability if oxygen is excluded or restricted. Radiation may also affect the radiopharmaceutical itself, giving rise to ions, radicals, and excited states. These species may combine with one another and/or with the active species formed from water. Radiation decomposition may be minimized by the use of chemical agents that act as electron or radical scavengers. Radiochemical purity must meet compendial standards throughout the time of use until the stated expiration of the radiopharmaceutical.

Determination of radiochemical purity is typically a two-stage process: 1) the different chemical species are separated by paper, thin-layer, or column chromatography or other suitable analytical separation technique; and 2) the radioactivity content in each of the separated chemical species is measured with a suitable radiation detector and counting device. The ultimate confirmation of acceptable radiochemical purity of a radiopharmaceutical is its intended biodistribution after administration.

3.14 Radionuclidic Identity and Purity

"Radionuclidic identity" is a critical parameter in radiopharmacy because the radionuclide used determines the radiation dose to the patient, the biodistribution of the radiopharmaceutical, the quality of any images obtained, or the efficacy of any therapeutic preparation. Radionuclide identity can be established either by measuring the half-life or the energy of the radiations emitted by the sample.

The radionuclidic purity of a radiopharmaceutical preparation refers to the fraction of radioactivity attributable to the desired radionuclide in the total radioactivity measured. Hence, a radionuclidic impurity is the presence of an unwanted radionuclide. Radionuclidic purity is important for radiopharmaceuticals, because unwanted radionuclides may cause several undesired consequences:

- Radionuclidic impurities may cause the radioactive assay of the radiopharmaceutical to deviate from the prescribed amount.
- Radionuclidic impurities may deliver higher-than-desired, radiation-absorbed doses to various organs and tissues.
- In some situations, radionuclidic impurities may interfere with image interpretation (diagnostic accuracy).
- It should be remembered that radionuclidic purity will change with time and is generally specified as a percentage of the desired radionuclide's activity at the time of calibration or at the time of administration (e.g., molybdenum-99 in technetium-99m).

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The radionuclidic purity must meet compendial standards throughout the useful life of the radiopharmaceutical. In addition, the impurity(ies) themselves will decay. Consideration must be given to the acceptable minimum and maximum allowable times between expiration of the product and analysis for impurities.

Radionuclidic impurities commonly arise during radionuclide production relating to impurities in target materials, differences in the values of various competing production cross-sections, and different excitation functions of competing reactions at the energy of the bombarding particles. In the case of generator-produced radionuclides, some generator breakthrough of parent radionuclide typically occurs and represents a radionuclide impurity in the eluate of the daughter radionuclide.

Determination of radionuclidic purity is typically based on evaluation of radioactive emissions, the principal analysis of the gamma spectrum obtained from a sample of the product. For short-lived isotopes, the half-life measurement could be an appropriate approach to assess radionuclidic purity. In cases involving radionuclidic impurities that have long half-lives relative to the desired radionuclide, measurement of the radionuclidic impurities can be performed after a sufficient time delay to allow the desired radionuclide to fully decay. In cases involving radionuclidic impurities that have substantially higher energy gamma emissions relative to the desired radionuclide, measurement of the radionuclidic impurities can be performed after placing the product inside a properly calibrated radiation shield that affords differential attenuation of the gamma rays emitted from the desired radionuclide versus those emitted from the radionuclidic impurities. Positron-emitting radionuclides typically cannot be differentiated, because their emitted energy (511 keV) is the same for each radionuclide; thus, gamma-ray spectrometry is not a recommended prerelease test for radionuclidic identity for PET radionuclides. Some positron-emitting radionuclides have characteristic gamma-ray emissions in addition to 511 keV, which may be used for purposes of identification (e.g., germanium-69 and sodium-22). In any case, the appropriate instrument should be chosen to detect potential impurities and should be properly calibrated to accurately quantify any identified impurities.

3.15 Chemical Purity

The chemical purity refers to the fraction of the total chemical species present in the product as the specified chemical component(s). Hence, a chemical impurity is the presence of an unwanted nonradioactive chemical. Chemical purity is important for radiopharmaceuticals, because chemical impurities may cause undesirable consequences such as chemical interactions (e.g., precipitation) and toxic biologic effects.

Chemical impurities are typically associated with production procedures and may include contaminants from raw materials, synthetic byproducts, solvents, excipients, equipment, preparative or purification columns, and containers. For certain radiopharmaceuticals, chemical impurities may also be associated with generator breakthrough of resin material from the generator column (e.g., alumina) in the eluate solution.

Determination of chemical purity is generally not performed and reported as a single attribute. Rather, determinations of individual chemical impurities are performed and compared to specifications (limits) for the respective individual chemical impurities. Such determinations of chemical impurities use analytical techniques as appropriate and described in the individual radiopharmaceutical monograph.

3.16 Labeling

Individual radiopharmaceutical monographs indicate that the labeling is to include the date and time of calibration, the amount of radioactivity associated with the radiopharmaceutical expressed as total MBq (μCi or mCi) and concentration as MBq (μCi or mCi)/mL at the time of calibration, the expiration date (and time, if appropriate), and the statement, "Caution—Radioactive Material". The labeling indicates that in making dosage calculations, a correction is to be made for radioactive decay and also indicates the radioactive half-life of the radionuclide. Other labeling requirements may apply to biologics or articles intended for injection. Beyond-use dates of compounded preparations should be included as appropriate. Additional labeling requirements may be required by various regulatory agencies.

3.17 Naming Conventions for Isotopes

Various naming conventions exist for isotopes that are associated with radiopharmaceuticals and radioactive devices. For example, the name of an isotope may or may not use a superscripted value for the mass number. Superscripted values should precede the elemental symbol for the isotope, and non-superscripted values should follow the elemental symbol, preferably with a hyphen between the symbol and the mass number. Examples include ^{68}Ga and O-18, respectively. Square brackets should be used to denote a specific isotope when necessary within a chemical name, for example, 2-[^{18}F]fluoro-2-deoxyglucose. Individual radiopharmaceutical monographs use nonproprietary names assigned by the U.S. Adopted Names (USAN) Council, which use the elemental symbol followed by the mass number separated by a space, for example, *Thallous Chloride Tl 201 Injection*. Although exceptions to these conventions undoubtedly exist, efforts should be made to adopt standardized conventions for radiopharmaceuticals and radioactive devices that fall within the scope of this general chapter.

4. INSTRUMENTATION FOR DETECTION AND MEASUREMENT OF RADIOACTIVE EMISSIONS AND APPLICATIONS

4.1 Ionization Chambers

Radioactive materials are not readily detected by ordinary chemical or physical methods. Instead, detection methods for radioactive materials rely on the ionization of matter that results from the emitted radiation. The charge separation created during this process forms the basis of radiation detection systems, which may be based on the ionization properties of gaseous, liquid, and solid materials.

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An ionization chamber is an instrument that directly measures ions produced in a gas as the result of the interaction of radiation with the gas. The most common usage in nuclear medicine applications is as the detector used in a dose calibrator. The dose calibrator is an instrument used to measure the quantity of radioactivity in a radiopharmaceutical. The key component of a dose calibrator is an argon-filled chamber with an applied electrical potential that allows the detection of ions produced by the passage of gamma rays through the chamber. Calibration of the system may involve one or more radionuclides with gamma energies and quantities that span the range of typical analyses. The calibration of the ionization chamber should be performed, when possible, with suitable NMI-traceable radionuclide standards. Routine system suitability testing should include checks for these parameters. Frequency of testing should occur as appropriate. Please refer to ⟨821⟩ for additional details on typical instrument requirements.

The position of the radioactive sample in the dose calibrator is ideal when it simulates 4π geometry. The geometric goal is placement of the sample at a point in the center of the cylindrical detector. Reproducibility of placement within the chamber is critical, because the response typically drops off at the top and bottom of the cylinder because of a combination of geometry and electronic effects. The value of the ionization current per unit of radioactivity, known as the "calibration factor", is characteristic of each gamma-ray-emitting radionuclide. The current produced in a dose calibrator is related to the mean energy of the emitted radiation and is proportional to the intensity of the radiation. The calibration of the dose calibrator for a specific radionuclide is ideally performed with a radioactive calibration source of the same radionuclide. Alternatively, it may be performed by measuring radioactive calibration sources with gamma energies above and below the gamma energy for the radionuclide to be measured and interpolating these values, also correcting for differences in gamma abundance, to establish the calibration factor for that radionuclide.

The upper limit of the dose calibrator is normally specified by the manufacturer. If not, testing is required to ascertain this upper limit. With a deep reentrant well-type chamber, reproducibility within approximately 5% or less can be readily obtained in a few seconds for quantities of radioactivity in the MBq (mCi) range and within about 30 s for quantities in the kBq (μCi) range.

The calibration of a dose calibrator should be maintained by relating the measured response of a standard to that of a long-lived reference standard, such as radium-226 in equilibrium with its daughters, cesium-137 in equilibrium with its daughters, barium-133, cobalt-60, or cobalt-57. The instrument should be checked on each day of use with the reference standard source to ascertain the stability over a long period of time. This check should include reference standard readings at all radionuclide settings used. Any necessary corrections for radioactive decay of the reference standard source should first be applied. It is also recommended that the reproducibility and/or stability of multi-range instruments be checked with the use of standards with appropriate activities for all ranges.

The size, shape, and location of a radioactive sample within the well will affect the response of a dose calibrator. This is usually referred to as "geometry". The shape, composition, and dimensions of the container holding the radioactive material can affect the result. Effects relating to container properties are generally more pronounced with radionuclides that emit beta particles (because of differences in Bremsstrahlung production) or emit low energy gamma or X-rays (because of differences in photon attenuation). It is important that geometric correction factors, if needed, be determined for each combination of radionuclide and configuration (i.e., size, shape, location with the well chamber, volume within the container, and container properties). The manufacturer's calibration factor for each radionuclide is determined using a specific geometry and container, which may not match the geometry or container used operationally.

4.2 Liquid Scintillation Counters

The liquid scintillation counter (LSC) detection method uses liquid scintillation cocktails to transform emitted radiation into detectable light photons. Alpha- and beta-emitting radionuclides may be assayed with the use of a liquid-scintillation detector system. In the liquid scintillator, the emitted radiation is converted into light quanta that are usually detected by two multiplier phototubes arranged to detect only coincidence radiation. The liquid scintillator is a solution consisting of a solvent, primary and secondary solutes, and additives. As the emitted particle dissipates energy in the solvent, a fraction of this energy is converted into fluorescent light by the primary solute. The function of the secondary solute is to absorb the primary fluorescence and re-emit the light at a longer wavelength that is more efficiently detected by the multiplier phototubes. Traditionally used solvents (cocktails) are toluene and *p*-xylene; primary solutes are 2,5-diphenyloxazole (PPO) and 2-(4′-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD); and secondary solutes are 2,2′-*p*-phenylenebis[4-methyl-5-phenyloxazole] (dimethyl-POPOP) and *p*-bis(*o*-methylstrylyl)benzene (bis-MSB). Many aqueous scintillating solution cocktails that are less hazardous are available. Aqueous solutions tend to have a shorter shelf-life; therefore, it is important to ensure that they have not expired before use. As a means of attaining compatibility and miscibility with aqueous samples to be assayed, additives, such as surfactants and solubilizing agents, are also incorporated into the scintillator. For an accurate determination of radioactivity of the sample, care should be exercised to prepare a sample that is truly homogeneous. Quenching is a major concern for liquid scintillation and refers to any mechanism that causes a reduction of emitted light by the source. Quenching can result from multiple factors, including oxygen and dilution effects; therefore, it is critical to perform quenching corrections by counting comparative measurements of standard samples using the same conditions of volume, additives, and solvent to accurately account for these effects.

Alternatively, an external source, typically barium-133 or europium-152, is placed in close proximity to the sample vial to release Compton electrons. The shape of the resulting spectrum is analyzed to compute a quench-indication parameter. This parameter can then be related to the counting efficiency by measuring sources of known radioactivity at a determined level of quenching agent. The resulting quench curve allows the determination of the radioactivity of an unknown sample knowing the count rate and value of the quenching parameter. The scintillation fluid may require special handling for disposal, in addition to any residual radioactivity. Static electricity on the vials may also cause spurious counts in the system, especially in the case where low-energy beta emitters are being assayed. This problem is often greater in a low-humidity environment.

The disintegration rate of a beta-emitting source may be determined by a procedure in which the integral count rate of the sample is measured as a function of the pulse-height discriminator bias, and the emission rate is then obtained by extrapolation to zero bias. Energetic alpha-emitters may be similarly measured by this method.

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4.3 Nuclear Spectroscopy Systems

GAMMA-RAY SPECTROMETRY

Each gamma-emitting radionuclide has a unique spectrum of mono-energetic photons emitted that allows the identification and quantification of radioactive materials in a sample by comparing the energy(ies) of the photon(s) detected and the intensity at each energy. This gamma spectrum allows for both the quantitative determination of purity as well as identity of the radionuclide. Gamma-spectrum analysis can be performed by using either a scintillation crystal, typically sodium iodide activated with thallium [NaI(Tl)], or by using a semiconductor detector consisting of a germanium–lithium (Ge–Li) crystal or a high-purity germanium detector (HPGe). Semiconductor detectors have a much higher energy resolution than NaI(Tl) detectors, with the ability to resolve gamma rays differing in energy by only a few keV, as opposed to the 20–80 keV required for a NaI(Tl) detector. Because of their increased resolution, semiconductor detectors are the preferred analytical method for gamma spectral analysis. A lanthanum–bromide detector is also available that has a significantly better resolution (10–12 keV) than the NaI(Tl) detector, without the necessary requirement of liquid nitrogen cooling for a HPGe detector. The use of spectroscopy software to automate the analysis is acceptable; however, the operator should have an understanding of the parameters chosen to ensure that the performance of the system is adequate to meet test requirements.

Semiconductor detectors are, in essence, solid-state ionization chambers, but the energy required to create an electron-hole pair or to promote an electron from the valence band to the conduction band in the semiconductor is about one-tenth the energy required for creation of an ion-pair in a gas-filled ionization chamber or proportional counter. This energy threshold is also far less than the energy needed to produce a photon in a NaI(Tl) scintillation crystal. The energy resolution is a measure of the ability to distinguish the presence of two gamma rays closely spaced in energy and is defined by convention as the full width of the photopeak at its half maximum (FWHM), expressed as a percentage of the photopeak energy. For example, with 1.33 MeV gamma rays from cobalt-60, a HPGe detector has an energy resolution of about 0.3% FWHM, whereas a 3-in × 3-in NaI(Tl) crystal has a value of about 6%.

Gamma-ray spectra exhibit one or more sharp, characteristic photopeaks, or full-energy peaks, as a result of total absorption in the detector of the full energy of incident gamma radiations. These photopeaks are useful for identification purposes. Other secondary peaks are observed as a consequence of backscatter, annihilation radiation, coincidence summing, fluorescent X-rays, and other factors, accompanied by a broad band known as the "Compton continuum", which arises from the scattering of photons in the detector and surrounding materials. Because the photopeak response varies with gamma-ray energy, calibration of the gamma-ray spectrometer should be achieved with radionuclide standards having well-known gamma-ray energies and emission rates from an NMI. The shape of the gamma-ray spectrum is dependent upon the shape and size of the detector, the types of shielding materials used, and the electronic processing characteristics of the instrument.

One of the most useful applications of gamma-ray spectrometry is the identification of radionuclides and the determination of radionuclidic impurities. When confirming the identity of a radionuclide by gamma-ray spectrometry and/or quantifying the radioactivity, it is necessary to ensure that the detector has been accurately calibrated using a known source, as described above, and in the same geometry as the unknown sample. Where the radionuclides emit coincident gamma or X-radiations, the character of the pulse-height distribution often changes quite dramatically because of the summing effect of these coincident radiations in the detector as the efficiency of detection is increased (e.g., by bringing the source closer to the detector); this is referred to as "cascade summing". Such an effect is particularly evident in the case of iodine-125. Most commercially available software packages include an ability to correct this source of error.

When identification of a radionuclide by means of a calibrated spectrometry system is not possible, the identity of the radionuclide may instead be established by measuring two or more of the following nuclear decay scheme parameters: (1) half-life; (2) energy of each gamma ray or X-ray emitted; (3) the abundance of each emission; and (4) E_{max}, the maximum energy of emitted beta particles, for those radionuclides that decay with beta-particle emissions. Such measurements should be performed as directed in ⟨821⟩. Agreement of two or more of the measured parameters within 10% of the corresponding published nuclear decay scheme data confirms the identity of the radionuclide.

As with other types of detectors, the background should be determined and subtracted from the measurement. In addition, the background should be stable, especially in situations where long counting times are required. This can be achieved by running a background spectrum before analysis and comparing it to a previously obtained background spectrum. Generation of a background spectrum will also allow the calculation of a minimum detectable activity for each possible impurity.

BETA PARTICLE COUNTING SYSTEMS

Beta (β) particles are emitted with a distribution of energies ranging from zero to a definite maximum value. The maximum energy of the electrons is characteristic of a particular radionuclide and is normally the E_{max} shown in nuclear data tables. The determination of the maximum beta energy may aid in the identification of the beta-emitting radioisotope, and careful measurements can routinely quantify activities. Emitted beta particles rarely possess the maximum energy. On average, emitted beta particles possess one-third of the maximum energy. Beta particles can be difficult to detect because they only penetrate small thicknesses of solid materials. [NOTE—Radioisotopes that emit gamma radiation in addition to beta radiation are more easily quantified and identified by using gamma-ray spectroscopy. In some cases, gamma-ray detection is the preferred method for measuring these radioisotopes and is usually the best means of identification of a beta/gamma-emitting radionuclide.]

Several detectors can be used for the detection and measurement of beta particles. These can be ionization chambers, proportional counters, and scintillation counters with their associated electronics. Self-absorption and backscattering can be an issue in beta-particle analysis and can result in a lower or higher number. Ionization chambers and proportional counters can be used for the quantitation of beta particles but are less suited for identification, because they cannot measure the maximum beta energy. Scintillation counters can be used for both the quantitation and identification of beta particles. When all or part of the energy of beta radiation is dissipated within scintillators, photons of an intensity proportional to the amount of dissipated energy are produced. These pulses are detected by an electron multiplier phototube and converted to electrical pulses, which are subsequently analyzed with a pulse-height analyzer to yield a pulse-height spectrum related to the energy spectrum of the

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incident radiation. In general, a beta-particle, scintillation pulse-height spectrum approximates the true beta-energy spectrum, provided that the beta-particle source is prepared in such a manner that self-absorption is minimized. Beta-particle energy spectra may be obtained by using calcium fluoride or anthracene as the scintillator. The spectra of charged particles may also be obtained using silicon semiconductor detectors.

The penetration power of beta particles is significantly larger than for alpha particles; a few millimeters of aluminum will stop beta particles. Care should be taken for the Bremsstrahlung X-rays that are created during the deceleration of the beta particles, because they may influence the measurement and can be a radiation safety concern.

Beta emitters can be quantitatively measured with ionization chambers and proportional counters that measure electrical current generated by the ionization of a select gas in a high-voltage field. Depending on the energy of the beta particle, the composition of the sample and container, and the design of the detector, the measurement may be based on Bremsstrahlung. For a radionuclide in solution, most of the beta energy is absorbed by the solvent, leaving only Bremsstrahlung exiting the sample. In the case of ionization chambers (e.g., dose calibrators), the walls housing the ionizable gas effectively convert all beta energy to secondary photons. As is the case for all radiation measurements, quantitative determinations with an ion chamber or a proportional counter depend on rigorous calibration and standardization of the sample type and geometry.

Depending on the detection setup, alpha particles can interfere with measurement of the beta particles. This can be easily prevented by positioning an absorber for alpha particles between the source and the measuring equipment. However, because low-energy beta particles (<200 keV) may also be absorbed, the count rate should be corrected for the absorption of these low-energy beta particles.

An LSC can also be used for the quantification of beta particles and aid in radionuclide detection. Because of the high efficiency of the method, LSC is particularly useful in measuring very low levels of radioactivity and is commonly used for complex biological samples. The sample is normally dissolved in a solution containing a phosphor that converts the beta emission to light pulses, which are detected by a very sensitive system of photomultiplier tubes. At energies greater than ~100 keV, the energy conversion and therefore the counting efficiency, is essentially 100%. The method is somewhat compromised by quenching effects, but these can be easily overcome with careful calibration.

When high energy (>800 keV) beta particles are measured, it is possible to count without scintillation cocktails, because the beta particles create Cherenkov radiation, which can be directly detected with a photomultiplier. [NOTE—Although alpha particles can interfere with beta counting, this problem can be corrected for by use of thin absorbers or by instrumental energy discrimination.]

The identification of pure beta-emitting radioisotopes without accompanying gammas is best done by a combined measurement of the half-life of the radioisotope and the maximum energy of the beta radiation emitted by the radioisotope.

The approximate maximum energy of a beta particle can be determined by two procedures: (1) by measuring the radioactivity as a function of absorber thickness; and (2) by plotting the logarithm of the count rate versus the thickness of the absorber in mg/m^2; then, an absorption curve can be made. The absorption curve can be compared with standardized absorption curves, which aids in the identification of the radioisotope.

Using LSC, by measuring the pulse height over a calibrated energy range, a beta-energy spectrum can be generated from which the maximum energy can be estimated. LSC instruments typically automate the calibration factor, but care should be taken in correcting for quenching effects.

ALPHA PARTICLE COUNTING SYSTEMS

Several detectors can be used for the detection and measurement of alpha particles. These can be ionization chambers, proportional counters, silicon semiconductor detectors, and scintillation counters with their associated electronics. For the identification and quantification of alpha particles, ionization chamber and proportional counters are not suitable.

Special precautions should be taken in the measurements of alpha particles because of their high energy but limited penetration power (about 40 µm in human skin).

For the identification and assay of alpha-particle emitters, spectrometry using liquid scintillation is mostly used. For the identification and determination of radionuclidic purity of alpha-particle emitters, spectrometry using a silicon-diode semiconductor detector can be used.

In a scintillation counter, the energy of the alpha particle is transformed to a light pulse, which can be detected by a photomultiplier tube. The intensity of the pulse is a measure of the energy of the detected alpha particle. Solid scintillation counters or phosphor detectors can be used as well as liquid scintillation.

When solid-state detectors are used, the sample is usually electroplated on a planchet disc, and the detector is brought close to or immediately on the sample. The counting efficiency of solid-state detectors tends to be low. In liquid scintillation counting, when the sample is dissolved in the appropriate medium, the efficiency can very high. Each method of counting has its own advantages and disadvantages. For solid detectors, advantages are lower background noise and a better differentiation between alpha and beta particles. Disadvantages are that no volatile samples can be measured, and self-absorption by the sample material (related to thickness of the sample layer) can lower the detected count rate, giving a falsely low outcome of the assay.

For liquid scintillation, advantages are the ease of sample preparation and no self-absorption, because the sample is mixed with the scintillation fluid. Disadvantages are higher background noise levels and poor separation between alpha and beta particles.

4.4 Detector Systems for Chromatographic Applications

Chromatographic applications, wherein the radioactive components of a mixture are separated based on their distribution between a stationary phase and a mobile phase, represent a unique usage of radiation detection systems. The most common chromatography applications include thin-layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (high-pressure liquid chromatography and high-performance liquid chromatography, typically denoted as HPLC). Depending on the chromatographic application and the type of emitted radiation, various detector systems may be used in the analysis of separated radioactive components.

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Gamma-ray detector systems can be used to dynamically measure separated radioactive components in GC and HPLC applications. In these applications, the eluate from the chromatography column is directed over or through the scintillating detector. Typically, these detector systems are not used for radionuclide identification; therefore, NaI(Tl) crystals tend to be the detector of choice in these applications. Depending on the required sensitivity of the technique, the geometry of detector may consist of a well that surrounds the eluate tubing, or a flow cell that passes the eluate across the surface of the detector. In either case, the design of the system should provide a reproducible geometric relationship between the tubing and the detector. In addition, the detector should be shielded sufficiently to prevent spurious peaks or baseline drift due to background radiation. For this application, the output pulses of the photomultiplier tube may be converted into an analog signal whose voltage is proportional to the number of pulses (i.e., the amount of radioactivity). In this manner, the resulting electric signal is used by a data acquisition and chromatography system to provide a chromatogram in a similar fashion as more traditional chromatography detection systems. The flow rate of the eluate and the amount of radioactivity in the eluate should be controlled to provide a count rate that is within the linear range of the detector system.

LSC methods can also be used in HPLC separations of beta-emitting components. Several techniques may be used in this application. In the first, the eluate from the chromatographic column is collected in discrete fractions. Each fraction is then mixed with the liquid scintillation cocktail before analysis in the LSC. Additional processing steps may be necessary before the addition of the cocktail to minimize quenching. In the second technique, the scintillation cocktail may be mixed with the eluate from the column before passage of the resulting mixture through an online flow cell surrounded by the photomultiplier tubes. Finally, inline solid scintillators may be used with in situ mixing of the eluate with a liquid scintillation cocktail.

Beta-particle detector systems can be used to measure separated radioactive components in TLC applications. In this application, windowless gas ionization detectors are most commonly used, and the detector is automatically scanned over the TLC plate, yielding a two-dimensional plot of the radioactivity. Because of the nature of the sample, self-adsorption is negligible, and the beta radiation is efficiently counted. If all of the components on the TLC plate contain the same beta emitter, the output signal is proportional to the fraction of radioactivity in the separated components. If the separated species contain different radionuclides, the detector should be calibrated with each radionuclide to correct for different signal responses. Alternatively, if such a detector is not available, the TLC plate can be cut into multiple strips and counted by an appropriate detector. The individual strips should be counted using the same geometry. For the highest sensitivity, the strips may be extracted with solvent and then counted by LSC.

GLOSSARY

Alpha particles (α): Positively charged particles that are emitted from nuclei during radioactive decay. Alpha particles are Helium-4 nuclei, consisting of two protons and two neutrons but no electrons.

Beta particles (β^-): Negatively charged particles that are emitted from nuclei during radioactive decay. Beta particles are electrons.

Bremsstrahlung: Electromagnetic radiation produced by the acceleration or especially the deceleration of a charged particle after passing through the electric and magnetic fields of a nucleus.

Calibration factor: The coefficient used to convert the measured ionization chamber current to a nominal radioactivity. This term is often referred to as the "calibration coefficient".

Calibration time: An arbitrary time at which the specified amount of radioactivity is present on a specific date.

Carrier free: A preparation free from stable isotopes of the same element as the radionuclide.

Counting assembly: An instrument that consists of a sensing unit and an electronic scaling device. The sensing unit may be a Geiger-Müller tube, a proportional counter, a scintillation detector in which a photomultiplier tube is used in conjunction with a scintillator, or a solid-state semi-conductor.

Dose calibrator (also referred to as radionuclide calibrator): A well-type ionization chamber commonly used to assay radiopharmaceuticals. Display units are typically in curies (μCi or mCi or Ci) or becquerels (kBq or MBq or GBq).

Gamma rays (γ-rays): Electromagnetic radiation emitted from nuclei during radioactive decay. Gamma rays have a wide range of energies. The gamma rays emitted from a given radionuclide are always at the same energy(ies), providing a unique signature that enables the identification of a gamma-emitting radionuclide.

Geiger-Müller counter (often referred to as a G-M counter or Geiger counter): An instrument in which a high-voltage potential is applied across a volume of inert gas for the purpose of collecting ions produced by a radiation field. The negative electrons are internally multiplied to produce a readily detectable current pulse. Display units are typically counts per minute (cpm) or milliroentgen per hour (mR/h).

Geometry: The characteristics of a radioactive source (i.e., container type, container wall thickness, volume and position of the container in the well chamber) that along with the physical characteristics of the ionization chamber affect the magnitude of the calibration coefficient for a specific radionuclide. The principal geometry considerations that may affect the accuracy of a source measurement in a dose calibrator are container configuration, source volume, position of the source in the chamber well, and the radionuclide itself. [NOTE—It is customary to compare a standardized preparation and radiopharmaceutical drug or preparation using identical geometry conditions for assay, identification, and other parameters. The validity of the result is critically dependent upon the reproducibility of the spatial relationships of the source to the detector and its surroundings and upon the accuracy of the standardized preparation.]

Ionization chamber: An instrument in which an electric field is applied across a volume of inert gas for the purpose of collecting ions produced by a radiation field. The positive ions and negative electrons drift along the lines of force of the electric field and are collected on electrodes, producing an ionization current. The most commonly used form of ionization chambers for measurement of the activities of radiopharmaceuticals is a well-type ionization chamber known as a dose calibrator.

Isobars: Nuclides with the same mass number (protons + neutrons).

Isomers: Atoms with the same number of protons and neutrons, but a different nuclear energy configuration. Short-lived radioactive isomers are generally referred to as metastable. Different isomers are different nuclides based on their nuclear energy configurations.

Isotones: Nuclides with the same number of neutrons and a different number of protons. Isotones are different elements with different atomic masses.

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Isotopes: Nuclides with the same number of protons and a different number of neutrons. Isotopes are the same element with a different atomic mass.

Isotopic carrier (also referred to as carrier)**:** A stable isotope of the element concerned either present in or added to the radioactive preparation in the same chemical form of the radionuclide.

Liquid scintillation counter (LSC)**:** An instrument that detects scintillation light from the absorption of radiation energy in a scintillation liquid. This instrument is used primarily for beta-emitting radionuclides that do not also emit gamma photons. For best results, the radioactive sample must be able to be dissolved in the scintillation liquid.

Minimum detectable activity: The smallest quantity of radioactivity that can be detected above the background with a specified level of confidence.

National Metrology Institute (also known as NMI)**:** A measurement standards body that is a laboratory of metrology that establishes standards for a country or organization. For example, the National Institute of Standards and Technology (NIST) is the NMI for the United States.

No carrier added: A preparation where no stable isotopes of the same element as the radionuclide being tested are intentionally added in the stated chemical form or at the position of the radionuclide in the molecule being tested.

Nuclide: An atom with a specific number of protons and neutrons in a given nuclear energy state.

Positrons (β^+)**:** Positively charged particles emitted from a nucleus during radioactive decay. Positrons are anti-electrons.

Radioactivity: (1) The spontaneous transformation of nuclei characterized by the emission of particles or photons. Radioactivity is typically described as atoms undergoing radioactive decay per unit time (or disintegrations per unit time). (2) The quantity of radioactive material, as measured in units of curies (U.S. units) or becquerels (SI units). The quantity of radioactive material may also be referred to as activity.

Radiochemical identity: The molecular structure of the intended active radioactive drug ingredient that is present in the radiopharmaceutical preparation.

Radiochemical purity: The ratio, expressed as a percentage, of the radioactivity of the intended active radiopharmaceutical ingredient to the total radioactivity of all radioactive ingredients present in the radiopharmaceutical preparation.

Radioisotope: A radioactive atom, generally used in the context of an isotope of an element.

Radionuclide: An unstable nuclide that undergoes radioactive decay; a radioactive nucleus. The terms radionuclide and radioisotope are commonly used interchangeably.

Radionuclidic identity: The intended radionuclide in the radiopharmaceutical preparation.

Radionuclidic purity: The ratio, expressed as a percentage, of the radioactivity of the intended radionuclide to the total radioactivity of all radionuclides in the radiopharmaceutical preparation.

Radiopharmaceutical (radiopharmaceutical preparation/radioactive drug)**:** A finished dosage form that contains a radioactive substance in association with one or more other ingredients and that is intended to diagnose, stage a disease, monitor treatment, or provide therapy. A radiopharmaceutical includes any nonradioactive reagent kit or radionuclide generator that is intended to be used in the preparation of any such substance. The terms radiopharmaceutical and radioactive drug are commonly used interchangeably.

Scintillation crystal counter: An instrument consisting of a crystal scintillator, such as NaI(Tl), with an attached photomultiplier tube and associated electronics. Scintillation light produced from the absorption of gamma and X-rays in the crystal is converted to electrons and amplified in the photomultiplier tube. The resultant current pulse may be further analyzed with regard to photon energy. A commonly used form of this instrument that has a hole in the crystal of sufficient size to allow placement of a test tube or similar container is known as a well counter.

Semiconductor detector: An instrument consisting of a semiconductor material, such as silicon or germanium crystals, that detects ionizing radiation through generation of charge carriers (passage of electrons through holes). The current pulse produced by migration of these charge carriers, under the influence of a voltage potential across the material, can be further amplified and analyzed to determine the quantity and energy of the incident radiation.

Solid-state detector: A crystal-based detector, in contrast to a gas-based detector; often is used as a synonym for a semiconductor detector.

Specific activity: The radioactivity of a radionuclide per unit mass of the element or compound. The unit of specific activity is radioactivity per mass expressed on a gram or mole basis [e.g., mCi/μg (MBq/μg); Ci/mmol (GBq/mmol)].

Strength: The radioactivity concentration of the radiopharmaceutical at the calibration time. The unit of strength is the amount of radioactivity on a volume basis (e.g., mCi/mL or MBq/mL).

Total radioactivity: The radioactivity of the radionuclide, expressed per unit (e.g., vial, capsule, ampule, generator, and others) at the calibration time.

Validation: Establishment of documented evidence that a method, process, or system meets its intended requirements.

Verification: Confirmation that an established method, process, or system meets predetermined acceptance criteria.

X-rays: A type of electromagnetic radiation emitted from the electron orbitals. Although they do not arise from the nucleus, they are often present immediately after a decay event if there are interactions between the emitted radiation and the orbital electrons.

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⟨1823⟩ POSITRON EMISSION TOMOGRAPHY DRUGS—INFORMATION

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1. INTRODUCTION

Positron emission tomography (PET) drugs contain radionuclides that undergo nuclear transformation, or radioactive decay, predominantly by the emission of a positron. Positrons undergo annihilation upon interaction with electrons to produce two photons that are emitted in nearly opposite directions to each other. Each photon possesses an energy of 511 keV, which lies in the gamma ray portion of the electromagnetic spectrum. These radionuclides are used in a wide range of PET imaging studies, including research, investigational, and clinical applications.

The radionuclides used in PET imaging studies typically possess short physical half-lives (denoted as $T_{1/2}$). Some common examples of PET radionuclides and their associated half-lives are included in *Table 1*. Note that *Table 1* includes radionuclides currently in predominant use and is not intended to illustrate all positron-emitting radionuclides used in PET.

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Table 1

PET Radionuclide	Half-Life, $T_{1/2}$
Fluorine-18	109.8 min
Carbon-11	20.4 min
Nitrogen-13	10.0 min
Oxygen-15	2.0 min
Copper-64	12.7 h
Gallium-68	68 min
Rubidium-82	75 s

Because certain of these radionuclides such as *Carbon-11* and *Nitrogen-13* when found in biological systems have the same chemical and physical properties as their stable counterparts found in biological systems, PET offers a unique platform for *in vivo* imaging studies of complex biochemical pathways. As a result, PET radionuclides have found widespread use in cardiology, oncology, and neurology applications. PET drugs have also attracted interest as potential tools to accelerate and reduce the cost of therapeutic drug discovery efforts.

Most PET radionuclides are produced at the point of use by a particle accelerator (e.g., a cyclotron) or a radionuclide generator. A cyclotron accelerates charged particles such as protons or deuterons to velocities sufficient to induce a nuclear transformation of the target nucleus into a different element. High-energy particles and/or radiation are emitted from the target nucleus during the transformation process. An example of a transformation is the bombardment of stable Oxygen-18 nuclei with accelerated protons to produce *Fluorine-18* nuclei along with the concomitant emission of a neutron. This process may be summarized according to the following shorthand notation:

$$^{18}O(p,n)^{18}F$$

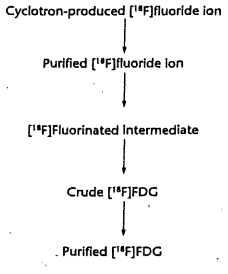
The chemical form and physical state of the bombarded nuclei vary, depending on the target and the subsequent usage of the PET radionuclide. This allows the production of PET radionuclides in gaseous, solid, and solution phases.

Radionuclide generators offer an alternative method for point-of-use access to some PET radionuclides. A radionuclide generator contains an immobilized parent radionuclide that undergoes radioactive decay to a daughter radionuclide, which may be eluted from the generator. An example of a PET radionuclide generator is the Germanium-68/Gallium-68 generator. The parent Germanium-68 ($T_{1/2}$ = 271 days) is adsorbed onto the column packing material. The Germanium-68 undergoes radioactive decay by electron capture into Gallium-68, which is not adsorbed onto the column packing material and may be eluted from the generator.

Regardless of the production method, other radionuclides may be present in addition to the desired PET radionuclide. In the case of cyclotron-produced PET radionuclides, other radionuclides may result from the bombardment of the target holder and/or other nuclei present in the target material. For generator-produced PET radionuclides, break-through of the parent radionuclide may occur. Safeguards are readily available to avoid unnecessary contamination with radionuclidic impurities of the final PET drug.

Once the PET radionuclide has been produced, it should be processed into a suitable form of a PET drug. The simplest form of processing involves the purification and/or formulation of the PET radionuclide. An example is the purification of the [^{18}F]fluoride ion. After production of the [^{18}F]fluoride ion by cyclotron bombardment, ion exchange procedures may be used to remove impurities and yield a formulation suitable for PET imaging studies. Another example is the purification of gaseous [^{15}O]oxygen through various solid supports before administration of the finished PET drug by inhalation.

Simple forms of processing are rare, because PET drug products are typically synthesized from the appropriate radionuclide through multiple synthetic processing steps. The exact process depends on the desired PET drug. The synthesis of 2-[^{18}F]fluoro-2-deoxyglucose ([^{18}F]FDG) provides an illustration. The first step in this synthesis is the preparation of the anhydrous [^{18}F]fluoride ion from cyclotron-produced Fluorine-18. The [^{18}F]fluoride ion is activated with a phase transfer catalyst such as tetraethylammonium or [2.2.2]-cryptand to enhance its reactivity toward nucleophilic substitution. The resulting complex reacts with mannose triflate to yield a fluorinated ^{18}F intermediate, which is deprotected and purified to yield [^{18}F]FDG. This process is summarized in the following set of equations:



In addition to the chemical processing and/or purification steps used to generate the desired radiochemical of interest, other steps are typically required to formulate the PET drug product. For injectable PET drug products, formulation steps may include

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dilution, addition of a stabilizer, pH adjustment, and other steps. The final formulation of the PET drug product should take place in a manner that minimizes the presence of bacterial endotoxins and bioburden. Finally, the product should be sterilized (e.g., by passage through a membrane sterilizing filter) to provide a solution suitable for intravenous administration. In the above example, [^{18}F]FDG becomes the drug product *Fludeoxyglucose F 18 Injection*.

Before use, the finished PET drug product should be tested to ensure that the product meets suitable standards of identity, strength, quality, and purity. Because of the short half-life of PET radionuclides, testing should be completed in a timely fashion; however, it is not possible to complete certain quality control (QC) tests within a suitable timeframe. In some cases, it may be necessary to adopt a "sub-batch" approach for QC testing, wherein a sub-batch of the PET drug product is prepared solely for purposes of QC testing. Examples of PET drug products with such short half-lives include [^{15}O]water and [^{13}N]ammonia.

Ultimately, the short half-lives of PET radionuclides create unique constraints for the production and testing of PET drug products and define how these products are used in research, investigational, and clinical settings. PET drugs are a unique class of products defined by the following characteristics:

- The mass of the radioactive ingredient in a PET drug product usually ranges from nanogram to microgram quantities. This affects pharmacological and toxicological considerations for PET drugs, usually by creating large safety margins due to low mass of the active ingredient in the administered dose.
- An entire batch of a PET drug product may be contained in a single vial. Samples withdrawn for QC testing are representative of the entire batch. PET drug products produced in this fashion undergo 100% QC testing.
- PET drug products are produced and handled in environments with overlapping areas of regulatory authority. For example, a site may be subject to requirements of any combination of the following authorities: Nuclear Regulatory Commission or Agreement State Agency, Food and Drug Administration, Environmental Protection Agency, occupational safety entities, etc. These agencies may have different and conflicting requirements of the manufacturing facility, some through regulation, some through guidelines published by the authorities, and some through license commitments made by the facility.
- PET drug products are generally produced at or near their point of use in small-scale facilities with limited personnel and resources. This requires:
  - Allowance for multiple operations in one area with adequate controls
  - Allowance for making and testing multiple PET drug products using shared equipment with appropriate cleaning between batches
  - Appropriate requirements for aseptic operations
  - Appropriate requirements for system suitability and other day-of-use activities
  - Appropriate QC requirements for components, materials, and supplies
  - Self-verification of significant steps in radionuclide production, PET drug production, compounding, and testing
  - Single-person oversight of production or compounding, testing, review of batch records, and release authorization
- PET drug products do not enter a traditional distribution chain. Instead, PET drug products require just-in-time deliveries typically performed by dedicated carriers with experience in handling radioactive materials.
- It is not possible to complete sterility testing for PET drug products before their use. Therefore, considerations are made to provide for the assurance of sterility for injectable PET drugs intended for human use.
- Procedures should be in place to notify the responsible individuals in a timely manner if a PET drug product is found to be in noncompliance after release for human use.

2. TECHNIQUES FOR PRODUCTION AND QUALITY CONTROL

The unique characteristics of PET drug products play a large role in the choice of instrumentation and techniques used in production and QC testing. Production techniques and analytical methods should be efficient and rapid. The selection of techniques and methods is also strongly influenced by the development stage of the PET drug. For example, early development efforts focus on radiolabeling, purification, QC methods development, and others. These efforts are designed to support the usage of the PET drug for *in vitro* studies, animal studies, and may even include first-in-human studies. At this stage, relatively small quantities of the PET drug are required at a limited number of institutions or geographic areas. Manual production techniques or semi-automated equipment may provide sufficient quantities to meet this demand. Analytical methods should also be suitable for the usage of the PET drug at the early stages of development. Typically, method development efforts focus on accuracy, precision, and linearity. Before the use of injectable PET drugs in human studies, provisions are made to ensure that bacterial endotoxins are controlled at suitable levels and that the product is sterile.

In later development stages, larger quantities of the PET drug may be required in more geographic areas. At this point, the synthesis, purification, and testing of the PET drug product should be well defined to support clinical trials. Development efforts at this stage typically focus on optimization and reliability of the production process. Manual production techniques are rarely used. Instead, semi-automated or fully automated techniques are typically required to provide sufficient quantities of the PET drug at multiple geographic locations. This approach simultaneously assures consistent quality attributes of the PET drug at multiple production facilities and minimizes radiation exposure of operators. Method development activities at this stage focus on specificity, ruggedness, robustness, and other characteristics required to support QC testing of the PET drug at multiple geographic locations.

Finally, at the commercial stage of production, semi-automated or fully automated techniques and equipment may be required to provide sufficient quantities of the PET drug in numerous geographic areas. Production techniques and analytical methods for commercial PET drug products should be well defined and adequately described in the appropriate marketing authorization.

Not all PET drugs are intended to advance through the various development stages from *in vitro* studies, to animal studies, to clinical trials, to commercial production. For example, a PET drug product may be produced at several institutions for research

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purposes without the intention of commercialization. In other instances, a PET drug product may be Food and Drug Administration (FDA) approved but is only available in a single geographic area. These factors often result in different strategies for production techniques, analytical methodologies, and the underlying studies and documentation to support these strategies.

3. QUALITY ASSURANCE

The goal of quality assurance (QA) is to ensure that the techniques and equipment used in production and testing result in a product that meets established standards for that specific PET drug product. The QA program should be sufficient to establish the reliability and suitability of the techniques and equipment as appropriate to the development stage of the PET drug product. The following topics should be considered in a QA program.

3.1 Reagents and Materials

Reagents and materials used in the synthesis and testing of a PET drug product should conform to established acceptance criteria. Procedures for procuring, receiving, testing, storage, and use of reagents and materials should also be considered. An audit trail may be established for the traceability of specific lots of reagents and materials to specific batches of product. These acceptance criteria, procedures, and the audit trail should be appropriate for the development stage of the PET drug product.

3.2 Change Control

Changes in the synthesis and test methods should be evaluated for their potential for altering the product quality and be approved in advance through established procedures. If the resultant PET drug product does not meet the criteria appropriate to the development stage, the process change is unacceptable. Acceptable changes should be appropriately documented.

3.3 Validation

Production and analytical test methods should be validated at a level that is appropriate for the development stage of the PET drug product. Validation of the production process should result in a process that is reliable and consistent. Analytical method validation demonstrates that a method can quantitatively measure a PET drug product reliably and reproducibly. It is not necessary to validate analytical methods that are described in USP compendia. The emphasis on validation typically increases as the PET drug product moves through the development stages toward commercialization. Validation of the computer software used to control automated production equipment should also be considered as appropriate.

QUALIFICATION

Equipment used in the synthesis and testing of PET drug products should be qualified as appropriate for the development stage of the PET drug product. This may include qualification procedures (installation, operational, and performance), as well as procedures for periodic maintenance and equipment calibration. For commercially available equipment, the equipment vendor may be able to support qualification requirements.

STABILITY STUDIES

Stability studies should be performed on PET drug products to establish suitable storage conditions and the expiration time and date. Quality attributes such as radiochemical purity, appearance, pH, stabilizer, preservative effectiveness, and chemical purity should be evaluated. The frequency and extent of these studies should be appropriate for the development stage of the PET drug product. Significant process changes require stability studies to be repeated.

4. PRODUCTION

4.1 Equipment for Manual Synthesis

Equipment intended for the manual synthesis of PET drug products may be based on apparatus found in a typical chemistry laboratory such as glass reaction vessels, heating blocks, tubing, and other items. These items should be suitable for their intended use and selected according to the needs of the process. Items that are reused and come into contact with the reactants and/or final drug product of the synthesis should be cleaned and depyrogenated as appropriate for the use of the final product.

Written procedures for the manual syntheses of PET drug products should contain sufficiently detailed steps to ensure that a reproducible process is followed for each batch by the operator. Observations of critical process parameters should be documented as appropriate in the batch record.

4.2 Equipment for Automated Synthesis

Preparation of *Fludeoxyglucose F 18 Injection* and other common PET drug products can be adapted readily to automated synthesis. The use of programmable controllers and/or computers with switches, solenoids, and sensors allows the operator to control the sequence of steps in an automated fashion. Thus, an automated synthesis requires minimal operator intervention, and batch-to-batch consistency is maintained with each step executed in a reproducible manner. Software may provide feedback to the operator such as alarm conditions, status reports, or an end-of-synthesis report. Modules for automated

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synthesis may be commercially available. These modules may use individual reagents or cassettes required for the appropriate PET drug product synthesis. The cassettes provide preassembled collections of reagents, reaction vessels, tubing, filters, and other items necessary to produce a batch of a PET drug product.

Laboratory robots may be appropriate in situations where complex manipulations are required for the procedure, such as the physical movement of a reaction vessel from one station to another, or the dispensing of doses from a vial into individual syringes. Customized equipment may also be fabricated to perform a specific function in the PET drug product synthesis.

5. QUALITY CONTROL

QC tests should be designed and executed in a manner that is appropriate for PET drugs. The short half-life of PET radionuclides significantly limits the timeframe for the performance of QC tests, and it may not be possible to complete all QC tests before the use of the PET drug product.

5.1 Quality Control Sampling

In most cases, the entire batch of a PET drug is contained within a single vial. The relatively small batch volume of PET drugs limits the amount of material available for QC testing. This is different from traditional manufacturing practices, where a batch may consist of numerous vials and not all vials are sampled for purposes of QC testing. QC sampling of PET drug products use a larger proportion of the drug volume, and thereby offers a greater degree of assurance that quality-related problems will be identified in the QC testing process.

5.2 Reference Standards

Reference standards are necessary to identify the API and impurities in the PET drug product. Reference standards may be commercially available or prepared in-house. Commercial reference standards should include documentation from the supplier to ensure they are properly characterized. The characterization and qualification of reference standards should be appropriate for the development stage of the PET drug product.

5.3 Conditional Release

When a required QC test for a PET drug product cannot be completed because of a malfunction of test equipment, it may be appropriate to conditionally release the batch. It is not appropriate in the absence of critical QC tests to conditionally release batches (e.g., radiochemical identity and purity). Procedures should be developed to describe the conditional release process, including the role of historical data, other QC test results, sample retention, and other procedures.

5.4 Out-of-Specification Results

When the results of a QC test do not meet product specifications, an investigation should be conducted to determine if the result is due to an analytical error (false positive) or if the result is due to a product failure. Because of the short half-life of PET radionuclides, it may not be possible to conduct investigations in the same manner as in traditional drug testing laboratories.

6. ANALYTICAL METHODOLOGIES

Various analytical methodologies and chromatographic techniques may be used for the testing of PET drug products. The most commonly used methodologies are discussed in this section for purposes of illustration. Similar principles should apply to other methodologies not included in this section.

6.1 Thin-Layer Chromatography

Thin-layer chromatography (TLC) involves separation, identification, and quantification of analytes using a stationary phase on a support or plate and a developing solvent that may also be known as mobile phase. The stationary phase may be silica or alumina spread in a uniform layer onto a plate of glass, metal, or plastic. A small sample of the PET drug product is applied (spotted) onto the stationary phase, and the plate is developed in a chromatographic tank. The mobile phase moves through the stationary phase by capillary action, and the analytes are separated based on partition, ion exchange, and/or adsorption. The retardation factor (R_f) and radiochemical purity of the PET drug on the developed plate can be determined by either counting the developed plate on a radio–TLC scanner, or cutting the plate into pieces and counting each piece in a radiation detector. The R_f of an analyte, under specific TLC conditions, is considered an identifying characteristic of the analyte.

6.2 Gas Chromatography

Gas chromatography (GC) involves separation, identification, and quantification of volatile analytes using a stationary phase and a gaseous mobile phase. A small sample of the solution is introduced into the GC instrument, which vaporizes the sample for passage over the stationary phase that is immobilized within a GC column. As the gaseous mobile phase passes over the stationary phase, the analytes are separated based on their partition between the gas and stationary phases. A detector located at the exit of the column provides an electronic signal to produce the gas chromatogram.

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A GC instrument consists of a gas source, injection port, column, detector, and a data collection device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The gas source depends on the column and detector in use. The type of detector depends on the nature of the compounds analyzed. Typical detectors include flame ionization and thermal conductivity. In addition, radiation detectors may be used in GC analysis. Detector output is recorded over time, and the instrument response, measured as peak area or peak height, is a function of the amount present. The retention time of an analyte under specific GC conditions is considered an identifying characteristic. GC analysis is most typically used to quantify residual solvents in PET drug products but may also be used to determine radiochemical and chemical purity of the PET drug product formulation.

6.3 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is an analytical technique used to separate, identify, and quantify the components of a solution. HPLC separations are based on the interaction of the analytes between the stationary and mobile phases, which in turn leads to the retention of the analytes. The mechanism of interaction between the analytes and stationary phase may be partition, size exclusion, adsorption, and/or ion exchange chromatography. The retention time of an analyte under specific HPLC conditions is considered an identifying characteristic.

HPLC instruments consist of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device. The type of detector used depends on the nature of the compounds analyzed. In addition to mass detectors such as refractive index, ultraviolet, and conductivity, radiation detectors are also used in HPLC analysis. An HPLC system with a radiation and mass detector allows the simultaneous determination of radiochemical and chemical purity, retention time, and the subsequent identity of the PET drug product. This configuration also allows the determination of specific activity. Elution methods include gradient or isocratic methods with aqueous and/or organic buffers.

The calibration of a GC or HPLC instrument may be achieved by different means. One approach involves the creation of a calibration curve from a range of standards with known concentrations. The calibration curve may be used over a specified period of time for product testing. A second approach for calibration involves the creation of a single-point calibration at the beginning of each testing cycle. The results may be averaged and used to provide a calibration factor for product testing. Regardless of the approach for calibration, the tailing factor and chromatographic resolution (or column efficiency as appropriate) should be determined routinely as a part of system suitability. Routine system suitability testing should include checks for these parameters.

6.4 Radiation Detectors Used in Chromatographic Techniques

Radiation detectors used in TLC, GC, and HPLC for quantitative measurements should be calibrated when possible with suitable NMI-traceable radionuclide standards at appropriate frequency. Routine system suitability testing should be performed on the radiation detection system to ensure proper operation. Because of the nature of TLC, system suitability for a radio–TLC scanner should address uniformity, positional accuracy, detector linearity, and resolution. The calibration of radiation detectors should be repeated as appropriate.

6.5 Multichannel Analyzer

A multichannel analyzer (MCA) is an instrument used to obtain a spectrum of gamma rays emitted by a PET radionuclide. The key component of a MCA is an energy-sensitive detector. On the basis of the gamma spectrum of a sample, radionuclides present in the sample may be identified and quantified. Calibration of the system is typically performed with certified standards and may involve one or more radionuclides with gamma energies and quantities that span the range of typical analyses at appropriate frequency.

6.6 Ionization Chamber

An ionization chamber, often referred to as a dose calibrator, is an instrument used to measure the quantity of radioactivity in a PET drug product. The key component of an ionization chamber is an argon-filled chamber with an applied electrical potential that allows the detection of ions produced by the passage of gamma rays through the chamber. Calibration of the system is typically performed with certified standards at appropriate frequencies and may involve one or more radionuclides with gamma energies and quantities that span the range of typical analyses.

7. QUALITY ATTRIBUTES

Quality attributes should be defined and assessed for each PET drug. These attributes should be suitable for the intended use of the PET drug and should also reflect the dosage form. Because most PET drug products are administered by intravenous injection, this section focuses on quality attributes associated with injectable PET drug products. Different attributes may be appropriate for other dosage forms. In addition, the definition and assessment of quality attributes may vary with the development phase of the PET drug product in comparison to the final drug phase.

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7.1 Appearance

Injectable PET drug products should be free of visible particulate matter. Because of the radioactive nature of PET drug products, the test for appearance should be a visual inspection that meets radiation safety requirements. The use of a visual standard should be considered to ensure the accuracy of appearance determinations.

7.2 pH

Injectable PET drug products should be in the pH range suitable for intravenous administration. Because of the limited volume of a PET drug product, pH measurement is typically performed using narrow-range paper strips. The use of pH standards should be considered to ensure the accuracy of pH determinations.

7.3 Total Radioactivity and Strength

The total radioactivity of a PET drug product may be determined by an ionization chamber and should be stated at a given date and time. From the total radioactivity and the volume of the PET drug product, the strength may be determined. The strength of the PET drug product should be stated, along with the date and time of the determination of total radioactivity.

7.4 Radionuclidic Identity

Half-life (also referred to as approximate half-life) is a characteristic of the radionuclide that may be used for its identification. To adequately confirm the identity of a PET radionuclide, the half-life should be measured in a suitable counting device over a period of time appropriate to the half-life of the radionuclide.

7.5 Radionuclidic Purity

By definition, all positron-emitting radionuclides emit 511 keV gamma rays but may also emit gamma rays with different energies. Therefore, it is generally not possible to determine the radionuclidic purity of PET drug products with a MCA. One suitable solution to this problem is the use of validation studies to ensure the removal and/or decay of accelerator-produced radionuclidic impurities during the production process. Other approaches may be appropriate depending on the characteristics and source of the radionuclidic impurities. In addition, periodic analysis of decayed samples in routine production with a MCA may be used to quantify radionuclidic impurities developed.

7.6 Radiochemical Identity and Purity

Depending on the physical and chemical properties of the PET drug product, the radiochemical identity and purity may be determined by TLC, HPLC, or GC. The identity of the active pharmaceutical ingredient (API), and possibly other analytes, should be based on the known retention time of the analyte. The simultaneous use of reference standards during sample analysis should be considered for purposes of radiochemical identification. The radiochemical purity of the PET drug product should be determined based on the sum of all chemical forms of the radionuclide of interest. As appropriate, it should be established during validation that all radiochemical analytes are eluting from the chromatographic system and that the radioactive detector is operating within its linear range.

7.7 Chemical Purity

The chemical purity of the PET drug product should be assessed for the presence of volatile impurities, residual reagents and/or precursors, and by-products. For example, residual solvents, such as acetonitrile and ethyl alcohol, may assessed by GC. [2.2.2]-Cryptand, a common reagent used in the preparation of PET drug products, may be assessed by colorimetric techniques.

7.8 Total Mass of the Active Pharmaceutical Ingredient and Specific Activity

Potential toxicity issues and pharmacological effects may be associated with the API in a PET drug product (e.g., the mass-dependent localization of neurotransmitters or other more generalized forms of toxicity). In these instances, the total mass of the API should be determined before use of the PET drug product. The total mass of the API contained in a patient dose should be defined for PET drug products where mass-related localization or toxicity concerns require such assessment, which may be determined by HPLC or GC. On the basis of the mass of the API, the specific activity may be calculated. To determine the appropriate quality of the injected drug product in the patient dose, any two of three measured parameters (total mass, total activity, or specific activity) are sufficient. Specific activity should be stated along with the date and time of determination.

7.9 Bacterial Endotoxin

The quantity of bacterial endotoxin in an injectable PET drug product should comply with USP standards (<175 USP Endotoxin Units/patient dose). The test for bacterial endotoxin uses a solution of limulus amebocyte lysate (LAL). Various methods exist for this test, including gel-clot, chromogenic, and others. Test samples should be obtained and handled in a manner that minimizes contamination before testing. In addition, control measures should be used to ensure accurate test results without interferences from the test solution, such as certain formulations that enhance or inhibit the interaction of LAL

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with bacterial endotoxin. This effect may be corrected by dilution of the PET drug product before testing. The necessity of dilution in this test is determined in the validation of the specific formulation of the PET drug product.

7.10 Sterility

Injectable PET drug products should be sterile. For reasons described in *1. Introduction*, it is not possible to complete the sterility test before the use of PET drug products. The test for sterility should consist of inoculating a test sample into media capable of supporting the growth of aerobic and anaerobic microbes, which may be established with an appropriate certificate of analysis. The test samples should be obtained and handled in a manner that minimizes contamination before testing. To reduce radiation exposure to operators, the test samples may be allowed to decay before inoculation. The inoculation should use techniques that minimize the potential for false positives during the sterility test. In addition, control measures should be used to ensure accurate test results without interferences from the test solution. After inoculation, the sterility test samples should be incubated for the appropriate period of time at the prescribed temperature. If visible growth is detected during the incubation period, the investigator or physician should be informed in a timely manner, and an investigation should be initiated to determine whether the growth was due to accidental contamination during the sample handling process or to a true product failure. The investigation should include identification of the microbe(s) in the sterility test sample to determine the source of the contamination. This aspect of the investigation may be limited if production and sterility test sampling occur in the same laboratory.

8. STERILITY ASSURANCE

A suitable sterility assurance program should be established for PET drug products that are intended for intravenous injection. This section describes appropriate measures that assure the routine production of a sterile product suitable for injection.

8.1 Sterile Membrane Filtration

Injectable PET drug products are typically sterilized by passage of the solution through a sterile membrane filter into a presterilized vial. To provide the greatest assurance of sterility, the PET drug product should pass through an appropriate sterilizing filter into a presterilized vial that has been assembled using aseptic techniques.

8.2 Aseptic Techniques

Critical steps that affect the sterility of the PET drug product should be identified and, where appropriate, aseptic techniques should be used to complete these steps. Aseptic techniques should include the use of a suitable gowning, proper handling of components, environmental controls, and others. Aseptic techniques should be described in written procedures.

8.3 Presterilized Components

PET drug production typically employs various components and containers that are presterilized and pyrogen free. Appropriate sterile components are generally assembled aseptically in a controlled environment before use in production. Presterilized vials and sterile diluents such as sterile water for injection or sodium chloride solution are typically used. Other sterile components are sometimes utilized in nonsterile applications in production where a commercially available product may be useful. An example is use of a sterile transfer line for fluid transfer prior to a sterilizing filter, where the diameter or length of a commercially available sterile line is appropriate to the application.

8.4 Environmental Controls

The environment where aseptic techniques are executed should be controlled to ensure appropriate aseptic conditions. These controls may include temperature and humidity, ventilation and air filtration, cleaning and disinfection, equipment maintenance, proper garb, and microbiological monitoring. Air filtration standards should conform to local and/or national standards relevant to environmental standards, for example, International Organization for Standardization (ISO) standards.

8.5 Media Fills

A media fill, also known as a "process simulation", is the performance of an aseptic procedure using a sterile microbiological growth medium in place of the PET drug product. The goal of a media fill is to test whether the aseptic procedure is adequate to prevent microbiological contamination during the actual process. Media fills may be used to evaluate aseptic techniques used in the assembly of presterilized components and to qualify operators for aseptic techniques. A media fill should be designed to ensure that the simulation is representative of the aseptic manipulations performed during the actual process, including personnel, components, gowning, locations, batch size, number of replicates, and other factors. After completing the media fill, components filled with media should be incubated appropriately to permit the growth of microbes. The use of negative controls should be considered during the media fill procedure.

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8.6 Suitability of Media

Sterility test media should be tested before use to ensure that the media adequately support the growth of microbes. These tests are typically referred to as growth-promotion tests and may be conducted in-house, by the supplier, or by a contract laboratory. Media should be used within the manufacturer's expiration date.

8.7 Suitability of the Sterility Test Method

The sterility test should be tested to ensure that the PET drug product is not bacteriostatic or fungistatic. The suitability of the sterility test may be conducted in-house or by a contract laboratory.

8.8 Membrane Filter Integrity Test

To ensure the proper sterilization of the PET drug product by passage through a membrane sterilization filter, the bubble point of the filter should be determined after completing the filtration process. The bubble point of the filter, measured in pounds per square inch (psi), is the pressure required to force air through the pores of the wetted membrane in the device. Other suitably validated procedures may be used.

8.9 Operator Training and Qualification

Operators involved in aseptic techniques should be trained in proper gowning, environmental controls, handling sterile components, and other techniques. Operators should be qualified through successful completion of media fills. Media fills should be periodically repeated to ensure ongoing competency of the technique.

9. LABELING

The labeling associated with PET drug products may evolve as the drug progresses through the various development stages. For example, the labeling for PET drug products used for *in vitro* and animal studies may be very simple and designed to avoid mix-ups in the routine use of the PET drug product. In later development stages, the labeling may include information required for the investigational use of the PET drug product in humans. Finally, at the commercial stage of production, the labeling should include ingredients, warnings, approved indications, and other elements required by the appropriate regulatory agencies (e.g., FDA, Nuclear Regulatory Commission, and others).

GLOSSARY

The following definitions apply to words and phrases as they are used in this chapter:

Accuracy: The closeness of test results obtained by that method to the true value established across the range of the method.

Active pharmaceutical ingredient (API): A radioactive substance that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is incorporated into a PET drug product to furnish a direct effect in the diagnosis or monitoring of a disease or a manifestation of a disease in humans, or monitoring treatment of disease or therapeutic procedures (e.g., tumor therapy). Both radioactive and nonradioactive forms of the PET drug are included in the API.

Batch: A quantity of PET drug product that is intended to have uniform character and quality, within specified limits, and that is made in a single, defined operational cycle.

Chemical purity: The purity of a PET drug product based on the nonradioactive components of the formulation, for example, residual solvents and/or volatile impurities, reagents, and or precursors used in the synthesis and purification, stabilizers, excipients, or by-products produced in the synthesis.

Compounding: The process of synthesis or formulation of a PET drug for use under the practice of pharmacy and medicine.

Conditional final release: A final release for patient administration before completion of a required test because of a breakdown of analytical equipment.

Limit of detection: The lowest amount of analyte in a sample that can be detected but not necessarily quantified under the stated method conditions.

Linearity: The ability to elicit test results that are, directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Lot: A quantity of materials (e.g., reagents, solvents, gases, purification columns, and other auxiliary materials) that have uniform character and quality within specified limits and are used to make a PET drug product.

Lower limit of quantification: The lowest amount of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated method conditions.

National Metrology Institute (NMI): A measurement standards body that is a laboratory of metrology that establishes standards for a country or organization. The National Institute of Standards and Technology (NIST) is the NMI for the United States.

PET drug: A radioactive substance (active pharmaceutical ingredient) that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is incorporated into a PET drug product to furnish direct effect in the diagnosis or monitoring of a disease or a manifestation of a disease in humans, or monitoring treatment of disease or therapeutic procedures (e.g., tumor therapy).

PET drug product: A finished dosage form that contains a PET drug, whether or not in association with one or more other ingredients.

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Precision (as repeatability): The degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample within a lab over a short period of time using the same analyst with the same equipment.

Quality assurance (QA): A planned and systematic program to ensure that a PET drug product possesses the quality required for its intended purpose.

Quality control (QC): A system for testing the quality of components, materials, supplies, and PET drug products by procedures, tests, analytical methods, and acceptance criteria.

Radiochemical identity: The molecular structure of the intended active radiopharmaceutical ingredient that is present in the radiopharmaceutical preparation.

Radiochemical purity: The ratio, expressed as a percentage, of the radioactivity of the intended active radiopharmaceutical ingredient to the total radioactivity of all radioactive ingredients present in the radiopharmaceutical preparation.

Radionuclidic identity: The intended radionuclide in the radiopharmaceutical preparation.

Radionuclidic purity: The ratio, expressed as a percentage, of the radioactivity of the intended radionuclide to the total radioactivity of all radionuclides in the radiopharmaceutical preparation.

Range: The interval between the upper and lower levels of a quality attribute that can be determined with a suitable level of precision and accuracy.

Reagents and materials: A reagent is a chemical used in the synthesis and/or testing of a PET drug, whereas a material is an ancillary object, such as tubing, glassware, vials, and others.

Retardation factor (TLC): The ratio of the distance the analyte moved from the origin line divided by the distance the solvent moved from the origin line denoted by the variable R_f.

Retention time (HPLC or GC): The time required, after the injection, for the analyte to move through the column and reach the detector.

Robustness: The measure of the capacity of an analytical method to remain unaffected by small variations in method parameters.

Ruggedness (as reproducibility): The degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different labs, different analysts, different instruments, different lots of reagents, different assays, and different days.

Specific activity: The radioactivity of a radionuclide per unit mass of the element or compound. The unit of specific activity is the amount of radioactivity on a mass basis [e.g., mCi/μg (MBq/μg) or Ci/mmol (GBq/mmol)].

Specificity: The ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and matrix components.

Strength: The radioactivity concentration of the active pharmaceutical ingredient in a PET drug product at a given date and time. The unit of strength is the amount of radioactivity on a volume basis [e.g., mCi/mL (MBq/mL)].

Sub-batch: A quantity of PET drug product having uniform character and quality, within specified limits, that is produced during one succession of multiple irradiations using a given synthesis or purification operation. A group of sub-batches collectively forms a batch that is intended to have uniform character and quality, within specified limits. Sub-batches may be required for PET drug products with very short-lived radionuclides (e.g., ^{13}N and ^{15}O), because QC tests cannot be completed before use.

System suitability: Requirements used to verify that the system performs according to established criteria.

Tailing factor (symmetry factor): A measure indicating the non-ideality of a chromatographic peak resulting from the distribution and the migration of the analyte through the chromatographic column.

Validation: Establishment of documented evidence that a method, process, or system meets its intended requirements.

Verification: Confirmation that an established method, process, or system meets predetermined acceptance criteria.

Add the following:

▲⟨1850⟩ EVALUATION OF SCREENING TECHNOLOGIES FOR ASSESSING MEDICINE QUALITY

1. INTRODUCTION
2. APPLICATION OF THE TECHNOLOGY
3. GENERAL INFORMATION ABOUT THE TECHNOLOGY
    3.1 Specifications, Relative Cost, and Data
4. PERFORMANCE EVALUATION
    4.1 Technology Applications and Analytical Performance Characteristics to Evaluate
5. FIELD EVALUATION
    5.1 Access, Handling, Maintenance, and Repair
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1. INTRODUCTION

The proliferation and spread of substandard and falsified (SF) medical products has been and continues to be a growing global concern. Examples have been reported on a range of SF medicinal products, including but not limited to essential medicines. These products threaten global public health by jeopardizing patient safety, adding to the cost of care, diminishing confidence in health workers and systems, increasing the risk of treatment failure, wasting valuable resources, and contributing to the development of drug resistance.

Fortunately, many analytical technologies and tools exist to evaluate product quality within the laboratory. In general, these technologies are well understood and characterized. Over the last decade, many of these tools have been miniaturized for portability to help combat the growing proliferation of SF medical products. One of the benefits of the field-deployable platform of these technologies is that it facilitates the screening of samples as part of a risk-based testing approach.

The capabilities of the portable screening technologies complement, but do not obviate the need for, laboratory-based technologies and their confirmatory power. Portable tools may help conserve the limited resources of these laboratories and drive a sustainable "work smart" approach to ensuring medicine quality by utilizing complementary methodologies of progressively increasing complexity to rapidly and reliably analyze large numbers of samples. The following examples describe where and how these technologies are currently used or may be used in the future:

- Manufacturing controls
- Supply chain screening
- Border control
- Customs inspection
- Post-market quality surveillance or regulatory monitoring
- Point-of-care screening

However, the potential and actual capabilities and limitations of many of these portable screening tools, and especially their performance in field settings, have not been established. To address this need, this chapter provides the structure and suggested requirements for performing an appropriate and pragmatic review of a given technology. Such a review would typically comprise general information, a performance evaluation, and a field evaluation. The information garnered from the review can then be used to inform selection, procurement, and deployment of the technology of interest. Within this chapter, the term technology refers to screening instruments, solutions, technologies, or tools that provide basic information about the authenticity or quality of a drug substance or product.

2. APPLICATION OF THE TECHNOLOGY

The first and most important aspect of an evaluation is to identify the target application of the technology. A clear understanding of the intended application of any technology will help identify the parameters that need to be evaluated and inform the decision as to whether multiple screening technologies might be needed in a particular setting. For example, some screening technologies evaluate non-chemical features of a product, such as appearance, packaging, labeling, and origin. The technology will need to correctly identify a falsified medicine, but it will not need to determine the level of chemical impurities within the drug product.

Furthermore, the intended application of the technology comprises that of both the original equipment manufacturer (OEM) and the user. If these applications align, then the initial part of an evaluation should involve reviewing the technology against the manufacturer's claims. However, if these applications do not align, further steps should be taken to evaluate the technology against the user's requirements and intended application. Intended applications may vary based on factors such as:

- Medicine types
- Anticipated users
- Geographic locations
- Points within the supply chain
- The public health consequences related to poor-quality medicines within supply chains

3. GENERAL INFORMATION ABOUT THE TECHNOLOGY

The first step of a review, prior to implementing the performance and field evaluations, is to acquire general information about the technology of interest. This should include, but is not limited to, answering the questions listed below.

3.1 Specifications, Relative Cost, and Data

SPECIFICATIONS

Questions regarding specifications include:

- Which model or version of the technology is being reviewed?
- What technique (e.g., Raman spectroscopy, infrared spectroscopy, chromatography) does the technology employ?
- What are the dimensions (i.e., size, weight) of the technology?
- What type of power source (i.e., electricity, battery, solar) is needed to run the technology?

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- What additional power requirements (i.e., converters, power conditioners, uninterrupted power supply) are involved?
- Are there any safety precautions that should be taken into consideration before using the technology (i.e., lasers, heat generating)?
- Is waste generated when using the technology? If so, what kind of waste, and how is it safely handled?
- How easily can the technology's equipment be cleaned?
- What additional resources (i.e., chemicals, water, electricity) are required to clean the technology?

RELATIVE COST

Questions regarding relative cost include:

- What is the up-front cost of the technology?
- What are the recurring costs and how much are they (e.g., consumables, maintenance costs)?
- What is the cost of performing a test?
- Will the technology and intended methods be utilized with enough frequency to offset the cost?
- What warranties are available when purchasing the technology, and how comprehensive are they?

DATA

Questions regarding data include:

- What alternative languages are available for the hardware/software?
- Does the technology have a readily accessible user manual?
- Does the technology need or have internet connectivity capabilities?
- What format are data files in?
- Can data be transferred between devices?
- Can data be transferred to external sources?
- How can the data be displayed?
- Can the data be easily summarized into a report that supports decision-making?
- Can the software capabilities maintain data integrity?
- What are the levels of software permissions (i.e., administrator, user, guest), and what level of access do they provide?
- Does the instrument have a usable bar or Quick Response (QR) code reader?

It is important to note that some of these questions complement and supplement those listed in *5. Field Evaluation*. Generally speaking, the questions listed in that section can be answered through the review of publicly available information on the technology of interest or discussion with the OEM.

4. PERFORMANCE EVALUATION

The analytical performance portion of an evaluation may include parameters discussed in various guidances and standards on method development and validation. These include *Validation of Compendial Procedures* ⟨1225⟩ (*1*); *Analytical Instrument Qualification* ⟨1058⟩ (*2*); the Food and Drug Administration (FDA) Guidance for Industry, *Analytical procedures and methods validation for drugs and biologics* (*3*); and the International Council for Harmonisation (ICH) (harmonised tripartite guideline), *Validation of analytical procedures: text and methodology Q2(R1)* (*4*). However, except for ⟨1058⟩, these references are geared toward the validation of a method; their application in the context of this chapter should therefore focus on the performance qualification of a specific instrument rather than a procedure.

4.1 Technology Applications and Analytical Performance Characteristics to Evaluate

This section provides potential reviewers with the performance characteristics to evaluate, depending on the intended applications of a given technology. Ideally, this analytical performance evaluation will take place in a laboratory where variables can be controlled and a confident assessment can be made regarding the technology's analytical capabilities and whether they align with the reviewer's needs. With the exception of *Application I*, below, the performance evaluation should also encompass a comparison of the analytical results of the screening technology with those of a well-accepted confirmatory or compendial procedure (e.g., high-performance liquid chromatography) using reference material or standards. This will help inform the application and implementation of the technology within a broader quality assurance and quality control system.

Table 1, *Table 2*, and the *Applications* below, have been adapted from ⟨1225⟩ and tailored to the context of this chapter. However, they provide a broad guide and should be adapted to fit the needs of the user and the capabilities of the technology of interest. Definitions of the listed characteristics are included in the *Glossary*.

QUALITATIVE APPLICATIONS

Application I: Verification of packaging, labeling, origin, and appearance of the sample or drug product
Application II: Identification of bulk drug substances or active pharmaceutical ingredients (APIs) in finished pharmaceutical products
Application III: Identification of contaminants or impurities in bulk drug substances or finished pharmaceutical products

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Table 1. Qualitative Analytical Characteristics to Evaluate

Analytical Characteristic	Application I	Application II	Application III
Specificity	Yes	Yes	Yes
Detection limit	No	No	Yes

QUANTITATIVE APPLICATIONS

Application IV: Quantification of major components of bulk drug substances or APIs in finished pharmaceutical products
Application V: Quantification of contaminants, impurities, or adulterants in bulk drug substances or finished pharmaceutical products
Application VI: Determination of product performance characteristics (e.g., disintegration, dissolution, drug release) in finished pharmaceutical products.

Table 2. Quantitative Analytical Characteristics to Evaluate

Analytical Characteristic	Application IV	Application V	Application VI
Accuracy	Yes	Yes	Yes
Precision	Yes	Yes	Yes
Specificity	Yes	Yes	Yes
Detection Limit	No	No	No
Quantitation Limit	No	Yes	No
Linearity	Yes	Yes	Yes
Range	Yes	Yes	Yes

5. FIELD EVALUATION

A field fitness evaluation of a screening technology should be conducted as a follow-up to a performance evaluation. This part of the review assesses the ability of a technology to operate effectively and efficiently in field settings, which may include, but is not limited to, resource-limited areas. Certain parameters should be assessed and certain questions answered. Although some of these parameters can be evaluated through online searches and discussion with the instrument manufacturers, other parameters will need to be evaluated in the field and this should be taken into consideration when planning a review. The parameters and questions listed in the section below are not exhaustive and some may not be applicable, depending on the technology. However, they provide baseline information necessary for making an informed decision about the field suitability of a given technology within a given country.

Determine whether the results provided by the technology when analyzing a medical product in the field correspond to results obtained from the performance evaluation in the laboratory. This does not mean that the exhaustive analytical testing done in the laboratory needs to be replicated in the field, but a smaller, pragmatic subset of samples and tests should be run to give the reviewer confidence that the technology does, indeed, function in a field setting.

5.1 Access, Handling, Maintenance, and Repair

Some questions to address include:

- Is the technology commercially available in the country?
- In countries where the technology is commercially available but needs to be imported, what are the marketing authorization requirements for importing the technology?
- What are the control requirements for exporting this technology out of its country of origin or distribution?
- Are there local service providers or authorized distributors in the country?
- Can the technology be maintained and repaired by trained users or is a local service provider required?
- If there is a local service provider, then where is it located and how can it be reached (i.e., in country office, via email, or by phone)?
- Are there other shipping requirements if there is a need to send the technology for calibration or repair?
- Are there additional resources required to calibrate, recalibrate, or repair the technology (i.e., internet access, reagents, standards, spare parts)?
- How accessible are these additional resources in terms of availability and affordability?
- How often are recalibration and performance maintenance of the technology required?

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5.2 Durability and Use

DURABILITY

Some considerations regarding durability of the technology include:

- What is the operating temperature range of the technology?
- How is the technology affected by humidity?
- How are results affected by changes in temperature or humidity?
- How tolerant is the technology of various changes in the operational environment?
  - Temperature
  - Humidity
  - Dust
  - Vibration
  - Electromagnetic interference
  - Light
  - Water
  - Electrical variability (e.g., voltage, surge, frequency)
- How tolerant is the technology of rough handling by users?
- Is the technology intrinsically safe and suitable for the intended environment (e.g., temperature, humidity)?
- Can the technology withstand a drop test [i.e., according to U.S. Military Standard [MIL-STD]-810G *(5)*, International Electrotechnical Commission [IEC] 60068 *(6)*, or ASTM D5276-98 *(7)*]?

USE

Some considerations regarding use of the technology include:

- Is the technology suitable to use for the intended application?
- What level of training (e.g., remote vs. in-person, time, complexity) is required to use the tool?
- What are the sample preparation requirements for analysis and is the testing destructive or non-destructive?
- What additional accessories, consumables, reagents, and standards are required for sample analysis?
- How long does it take to analyze a sample and how easily can the results be interpreted?
- How many samples can be run simultaneously?
- What offline data analysis is required to interpret the results?
- What offline data analysis is available for experienced users?
- What requirements are there for fleet management of multiple instruments?
- What types of products (e.g., tablets, injections) can the technology analyze?
- Can models and/or methods developed on another instrument be remotely transferred to field units as needed?

5.3 Protocol and Statistics

PROTOCOL

When evaluating a screening technology for suitability, the applicable sections of this chapter should be used to develop a robust, practical, and ideally, standardized protocol that specifically outlines the work to be done. After selection of the applicable sections, additional variables need to be taken into account to ensure that the evaluation generates evidence-based data that can inform decision-making. These variables include but are not limited to the following:

- Number of units of the screening technology needed to determine variability between instruments (any evaluation should include at least two units of the technology in question)
- Types of dosage forms (e.g., solid oral dosage forms, injectables) to use for the evaluation
- Types of APIs to use for the evaluation
- Number of batches per product to use for the evaluation
- Number of units per sample to use for the evaluation

STATISTICS

The prudent use of statistics as part of an evaluation is a critical component for ensuring the integrity and reliability of any data generated. When possible, the specific statistical approaches and methodologies to be used should be identified, and a professional statistician should be consulted to devise an appropriate protocol. Concomitantly, it is valuable to collect available information from the literature and specific OEMs about the statistics associated with various applications of the technology. This approach provides a strong baseline to expand upon.

The following statistical guidances, references, and standards can be used to help with planning statistical approaches and methodologies:

- *Analytical Data—Interpretation and Treatment* ⟨1010⟩ *(8)*

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- *Statistical Tools for Procedure Validation* ⟨1210⟩ (*9*)
- Annals of Internal Medicine Research and Reporting Methods—QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies (*10*)
- ASTM E2586-16 Standard practice for calculating and using basic statistics (*11*)
- BMJ Research Methods & Reporting—STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies (*12*)
- *Guidance for Industry and FDA Staff—Statistical guidance on reporting results from studies evaluating diagnostic tests* (*13*)
- International Organization for Standardization (ISO) 3534-2, *Statistics—vocabulary and symbols—part 2: applied statistics* (*14*)
- ISO 3534-3 *Statistics—vocabulary and symbols—part 3: design of experiments* (*15*)
- ISO 5725-1 *Accuracy (trueness and precision) of measurement methods and results—Part 1: general principles and definitions* (*16*)

GLOSSARY

For reference purposes, some definitions are based on ⟨1225⟩.

Accuracy: The accuracy of an analytical procedure is the closeness of the test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. [NOTE—The definitions of accuracy in ⟨1225⟩ and the ICH harmonized tripartite guideline, *Validation of analytical procedures: text and methodology* Q2 (R1) (*4*) correspond to unbiasedness only. In the *International Vocabulary of Metrology* and documents of ISO, "accuracy" has a different meaning. In ISO 5725, accuracy combines the concept of unbiasedness (termed "trueness") and precision, and is defined as the closeness of agreement between a test result and the accepted reference value.]

Adulterant: Any substance that has been (1) mixed or packed with a drug so as to reduce its quality or strength or (2) substituted wholly or in part for the drug.

Detection limit: The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

Linearity: ICH documents define linearity as the ability to obtain test results that are proportional to the concentration of analyte in the sample across a given range.

Precision: The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

Quantitation limit: The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable *Precision* and *Accuracy* under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

Range: The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of *Precision*, *Accuracy*, and *Linearity* using the procedure as written. The range is normally expressed in the same units as the test results (e.g., percent, parts per million) obtained by the analytical procedure.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation. Robustness provides an indication of the procedure's suitability during normal usage. Robustness may be determined during development of the analytical procedure.

Screening: The process by which a sample undergoes an initial assessment of quality to determine acceptability or the need for additional testing.

Specificity: ICH documents define specificity as the ability to identify the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components (*4*). [NOTE—Other reputable international authorities (e.g., International Union of Pure and Applied Chemistry, AOAC-International) have preferred the term "selectivity," reserving "specificity" for those procedures that are completely selective.] The definition of specificity, above, has the following implication for the tests discussed below:

1. Identification tests ensure the identity of the analyte.
2. Purity tests ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., heavy metals, organic volatile impurities).
3. Assays provide an exact result, which allows an accurate statement of the content or potency of the analyte in a sample.

Supply chain: The system of manufacture, distribution, and dispensing of pharmaceutical products.

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⟨1852⟩ ATOMIC ABSORPTION SPECTROSCOPY—THEORY AND PRACTICE

THEORY

The instrumental technique of atomic absorption spectrometry (AAS; see *Appendix* for a list of acronyms in this chapter) uses the Beer–Lambert Law (Beer's Law), which relates the concentration of an analyte in a sample to the sample's absorption of electromagnetic radiation. Beer's Law states that the optical absorbance of a chromophore in a transparent solvent is linearly proportional to the chromophore's concentration and also to the sample cell path length. Beer's Law is applicable only if the spectral bandwidth of the light is narrow compared to the spectral line widths in the spectrum and is expressed as follows:

$$A = \varepsilon bc$$

A = absorbance
ε = molar absorptivity [L/(mol · cm)]
b = path length of the sample cell (cm)
c = concentration (mol/L)

In addition:

$$A = \log_{10}(P_0/P) = \log_{10}(1/T) = \log_{10}(100/\%T) = 2 - \log_{10}\%T$$

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P_0 = intensity of light passing through a sample
P = intensity of light leaving the sample
T = transmittance
$\%T = 100 \times T$

AAS methods fall into two categories: flame (FAAS) and flameless. Flameless methods include graphite furnace AAS (GFAAS), also known as electrothermal vaporization AAS (ETVAAS), for ultra-trace analyses. Other flameless methods are cold vapor AAS (CVAAS), intended specifically for the analysis of mercury, and hydride generation AAS (HGAAS), intended specifically for the analysis of arsenic, bismuth, germanium, lead, antimony, selenium, tin, and tellurium.

A basic AAS consists of a radiation source, a sample introduction device, a means to atomize the sample, a monochromator or a polychromator, a detector, and some means of acquiring data (usually a computer). Most instruments also have some sort of background correction system, which will be discussed later in this chapter.

In the case of FAAS, the flame through which the sample passes is considered to be the sample cell. In the case of electrothermal vaporization (ETV), the graphite tube into which the sample is deposited is considered to be the sample cell. In the case of CVAAS, a quartz absorption cell is mounted on top of the burner head, and the space above the burner in the optical path is considered to be the sample cell. In the case of hydride generation, hydrides are swept into a heated cell.

INSTRUMENTATION



Figure 1. Basic components of an atomic absorption spectrophotometer.

All atomic absorption spectrophotometers share fundamental components (*Figure 1*). A wide variety of AAS is available commercially and is based on one of two designs: a single-beam or a double-beam light path.

Figure 2 illustrates the light path for a single-beam spectrophotometer. The dotted line represents the modulated light signal from the external light source (line source), and the solid line represents the direct current signal from the atomizer, represented by a flame. The ovals represent lenses.

In this example, the beam from the line source is electrically modulated, and an amplifier placed after the detector is tuned to this modulation frequency. The beam from the line source becomes coded in this manner. The result is that the noise from the radiation emitted at all frequencies, except for the resonance frequency, is rejected, and the signal-to-noise ratio is improved.

Single-beam spectrophotometers rely on a very stable line source to maintain accuracy. It is also necessary to run a separate blank solution in order to provide a correct 100% T adjustment.

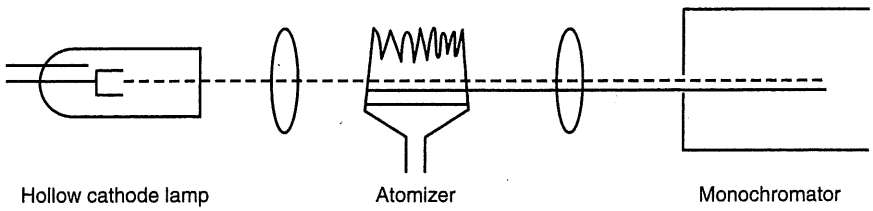

Figure 2. Schematic of a single-beam atomic absorption spectrophotometer. Reproduced with permission of the Royal Society of Chemistry.

Figure 3 illustrates the light path for a double-beam spectrophotometer. The radiation beam from the line source is divided by a mirrored chopper. One half is directed through the atomizer (sample beam), and the other half is directed around it (reference beam). Note that the beam from the line source is mechanically modulated by the chopper. The two beams are then recombined by a half-silvered mirror and are directed into a monochromator where the photons at the characteristic wavelength are measured by the detector. The ratio between the sample signal and the reference signal is amplified and processed as the absorbance reading. Because the sample and reference beams are generated from a common line source, are separated into their characteristic wavelengths by the same monochromator, and are processed by the same electronics, any variations in the radiation source, detector properties, or electronics are canceled out. In principle, the stability of a double-beam spectrophotometer is superior to that of a single-beam spectrophotometer.

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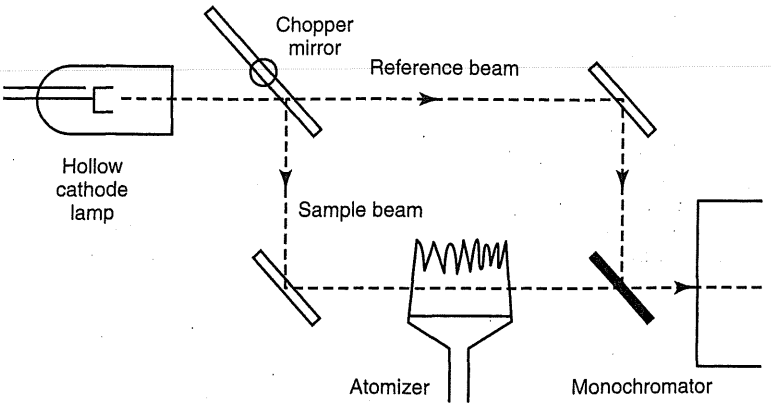

Figure 3. Schematic of a double-beam atomic absorption spectrophotometer. Reproduced with permission of the Royal Society of Chemistry.

SAMPLE CELL DESIGNS

Flame Atomic Absorption Spectrometry

In FAAS, a liquid sample is aspirated into a flame via a nebulizer. Via the nebulizer, the sample is converted to a mist that is composed of uniform droplets that are easily introduced into the flame. The flame desolvates and atomizes the sample providing a source of neutral atoms or molecules for analysis in the spectrophotometer.

Although other flame types have been documented, the most commonly used flame is an air–acetylene flame. Because the temperature of the air–acetylene flame is not sufficient to destroy oxides that might form or are present, a nitrous oxide–acetylene flame often is used, depending on the analyte and nature of the sample. The air–acetylene flame burns within a temperature range of 2125°–2400°, but the nitrous oxide–acetylene flame burns within a temperature range of 2650°–2800°.

Flames can be optimized for a particular analysis by either increasing or decreasing the fuel to oxidant ratio. The fuel to oxidant ratio can be adjusted to be either lean or rich—oxidizing or reducing—depending on the analyte of interest. Most instrument manufacturers provide guidance regarding the type of flame to use for a specific element, and analysts should refer to the manual provided by the instrument manufacturer for optimal flame conditions for a given analyte.

Some AAS can also be used in the flame emission mode. In flame emission, the atoms and molecules achieve an excited electronic state following thermal collisions within the flame, and upon their return to a lower or ground electronic state they emit light at characteristic wavelengths for each analyte. Although instrumentation may be capable of operation in the flame emission mode, this will not be discussed further in this chapter.

Electrothermal Vaporization—Graphite Furnace Atomic Absorption Spectrometry

In ETV or GFAAS, a liquid sample is deposited through a small opening into a graphite tube known as a mini-Massmann furnace. Inside the furnace, the sample is heated at increasing temperatures until the solvent is evaporated, solid residue is ashed or pyrolyzed, and neutral atoms are atomized in their ground states. The atoms are then excited by absorption of radiation at characteristic wavelengths. Samples can be deposited either directly onto the wall of the graphite furnace or onto a small graphite platform, known as a L'vov platform, which sits inside of the graphite furnace. With ETV, a series of heating steps is usually employed, including drying, charring or ashing, atomizing, and clean-out. Other intermediate heating steps can be used, depending on the nature of the sample. Throughout the heating process, the graphite furnace is purged with an inert gas, usually nitrogen or argon. At the atomization step, the furnace is quickly heated to a high temperature (usually to incandescence). The purge gas flow is stopped temporarily as a transient absorption signal produced by the atomized analyte is measured. As in FAAS, Beer's Law can be used to relate the concentration of the analyte and the absorption signal.

Cold Vapor and Hydride Generation Atomic Absorption Spectrometry

Cold vapor and hydride generation techniques are often used for the determination of mercury or for some hydride-forming elements such as tin, arsenic, selenium, antimony, and bismuth.

In the case of mercury, a chemical reduction generates atoms, and a stream of inert gas sweeps the cold vapor into a cold quartz cell in the optical path of the instrument. The technique is very sensitive and has detection limits that range from parts per billion (ppb) to parts per trillion (ppt) depending on the sample and the laboratory environment.

In the case of hydride-forming elements, a reaction with sodium borohydride and hydrochloric acid generates the hydride of the analyte of interest. The resulting gas is swept into an inert quartz cell that is positioned on top of the burner. The cell can be externally heated, or it can be heated by an air–acetylene flame. The heat of the flame breaks down the hydride and creates the elemental form of the analyte. This is known as the direct-transfer mode of hydride generation. Commercial direct-transfer hydride generators are available in two configurations, continuous flow and flow injection. As with the cold vapor detection of mercury, hydride generation also can be very sensitive and has detection limits in the ppb or ppt range.

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LINE SOURCES

An external light source (line source) is used to emit spectral lines corresponding to the energy required to elicit the electronic transition from the ground state to an excited state in the sample. The external light sources most commonly used are hollow cathode lamps (HCL) or continuum electrodeless discharge lamps (EDL). Absorption of radiation from the external light source is proportional to the population of the analyte species in the ground state, which is proportional to the concentration of the analyte that is aspirated into the flame, thereby making it possible to use Beer's Law to determine the concentration of an analyte in the sample. The absorption is measured by the difference in transmitted signal in the presence and absence of the analyte.

A suitable line source for AAS must:

- Produce lines of sufficiently narrow bandwidths specific to a particular atomic absorption peak
- Produce a beam of radiation of sufficient intensity to allow high signal-to-noise absorption measurements
- Produce a beam of radiation that is stable for extended periods of time
- Be easy to start and have a short warm-up time and an extended shelf life.

The gas contained within the HCL tube is ionized when an electrical potential is applied across the electrodes. Gaseous cations then acquire sufficient kinetic energy to dislodge some of the metal atoms from the anode surface, a process known as sputtering. A portion of the resulting cloud of metal ions is excited. Upon relaxation to the ground state, the ions emit photons at the characteristic wavelengths for that metal. HCLs are available in a variety of configurations and can be specific for a single element or multiple elements.

For certain elements, EDLs produce much more intense radiation beams than do HCLs. They are available for the analysis of antimony, arsenic, bismuth, cadmium, cesium, germanium, lead, mercury, phosphorus, selenium, tellurium, thallium, tin, and zinc. They are similar to HCLs because they rely on the sputtering of a metal by accelerated ions, but they ionize the inert gas by means of an intense radio-frequency field instead of hard-wired electrodes.

The requirement for an individual line source for each metal limits AA to a single-element technique. Some modern systems allow multi-element analyses by automatic lamp switching or by positioning multiple lamps in an array. High-resolution continuum source AAS combines a high-resolution Echelle monochromator with a Xenon short-arc lamp to obtain multi-element analytical capabilities (see *Reference 1* for additional details).

WAVELENGTH SELECTORS

Because atomic resonance lines are narrow, spectrometers are frequently equipped with monochromator gratings of moderate resolution, such as Ebert and Czerny–Turner monochromator systems.

Although the application is not common, high-resolution Echelle polychromators have been used in AAS designed for simultaneous measurements of multiple elements. Spectrophotometers of this type usually mate a high-resolution polychromator with either a Xenon arc continuum source or several individual line sources. They typically are equipped with a solid-state detector system.

DETECTION SYSTEMS

Detection systems convert radiant energy, photons, into a concentration-proportionate electronic signal. This signal is amplified and processed into an absorbance reading or a concentration, as illustrated in *Figure 1*. Photomultiplier tubes (PMTs) are widely used in AAS to convert photons passed through the monochromator into voltages. Some spectrophotometers are designed so that the applied PMT currents are under operator control. As mentioned above, some modern AAS are equipped with solid-state detection systems. There are two types: a charge-injection device or a charge-coupled device. Advantages and disadvantages of each design depend on specific applications. In some applications, a solid-state detector can produce a superior signal-to-noise ratio, provide a flatter response across the UV/Vis spectrum, and/or have improved background correction capabilities when compared to a PMT.

BACKGROUND CORRECTION

Nonspecific absorption can compromise the accuracy of AAS measurements. This is particularly the case for ultra-trace analyses using GFAAS. The type of background correction used for GFAAS can have a large impact on the success of the analysis. Three types of background correction designs are available for modern AAS: continuum source, Smith–Hieftje or variable giant pulse, and Zeeman effect.

Continuum Source

A continuum source, frequently a deuterium lamp, is configured in the spectrophotometer optics so that radiation from the continuum source and the line source is alternately passed through the sample cell by a beam chopper (*Figure 4*). The width of the continuum source bandpass is equal to the slit width.

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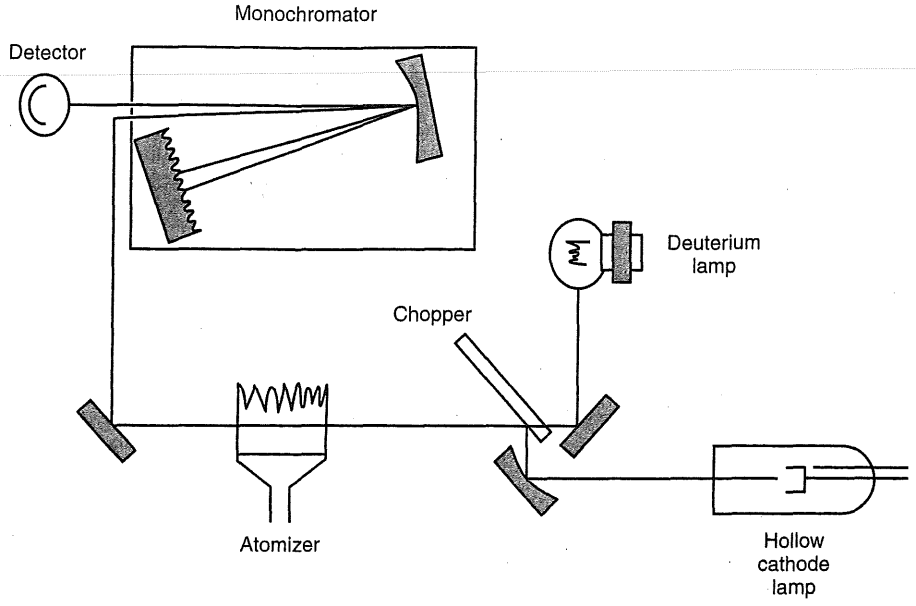

Figure 4. Schematic of an atomic absorption spectrophotometer with a continuum source background corrector. Reproduced with permission of the Royal Society of Chemistry.

The signal intensity from both sources can then be measured, and the ratio can be calculated.

Figure 5 displays the operation of a continuum-source background-correction system. Note that before the analysis, the intensity of the deuterium lamp and the HCL are equalized at the analyte's resonance wavelength. Also note that the bandwidth of the continuum source is equal to the width of the monochromator's exit slit, usually 0.2–0.7 nm, compared to the width of the resonance line of approximately 0.002 nm. If a solution containing the analyte of interest is introduced, the signals from both sources are attenuated because of atomic absorption (*Figure 5*, Panels A and B). If a solution containing the analyte of interest plus components that lead to nonspecific broad-band absorbance is introduced, the intensity of atomic absorption of the line source (HCL) is correctly subtracted from the intensity of the continuum source (*Figure 5*, Panels C and D).

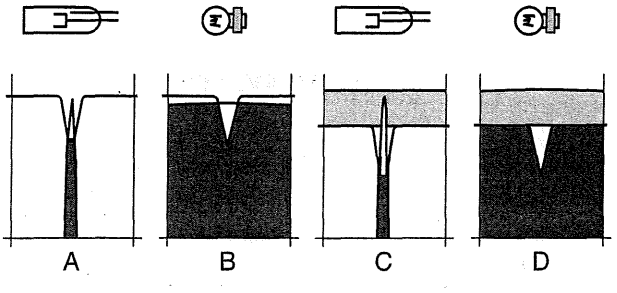

Figure 5. Panels A and B: atomic absorption only. Panels C and D: atomic absorption with background absorption. Reproduced with permission of the Royal Society of Chemistry.

Although continuum-source background correction is a useful design, it has some limitations. Basically, the introduction of an additional lamp and chopper causes a degradation of the overall signal-to-noise ratio of measurements. In addition, if the line source and the continuum source are not in perfect alignment, an erroneous correction will result when the individual beams are passed through an inhomogeneous gaseous sample, as this frequently occurs in the sample cell. This is particularly the case for highly structured backgrounds. The radiant intensity of the deuterium source in the visible region is too low to use for analytical wavelengths above 350 nm. Some instrument manufacturers equip their spectrophotometers with alternative continuum sources, such as tungsten halogen lamps, to remedy this issue.

Smith–Hieftje

When high currents are applied to an HCL, the emission-line profile is altered: the line is broadened and a dip appears in the center because of self-absorption. Self-absorption occurs when the photons emitted by excited atoms are absorbed by gaseous ground-state atoms contained within the quartz tube of the HCL. At currents used for normal data acquisition, the HCL is used to measure the sum absorbance of the element of interest and the nonspecific background. At high lamp currents, the measured absorbance is predominantly caused by the background. The absorbance caused by the element of interest is the difference between the intensities of analyte and nonspecific background absorbances. *Figure 6* illustrates the Smith–Hieftje background correction process.

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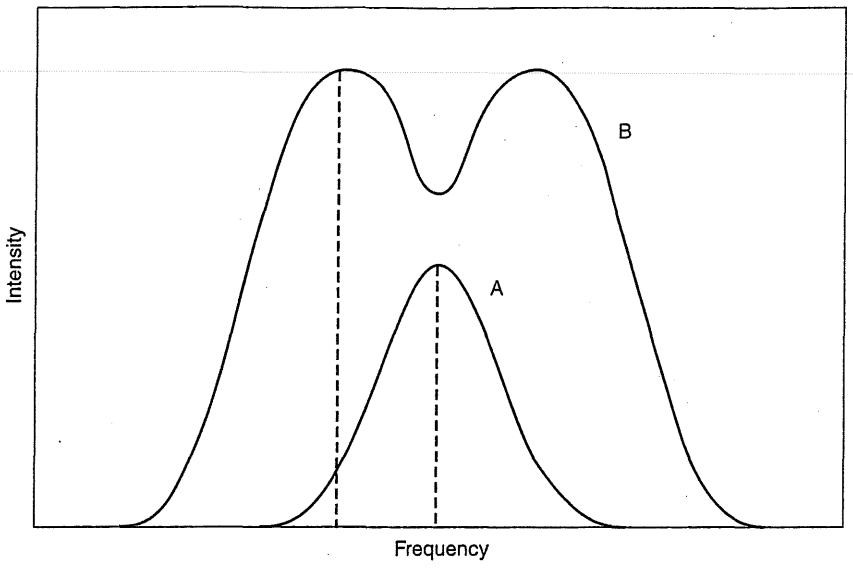

Figure 6. Emission profile of an HCL at (A) low current and (B) high current. Low current absorbance is caused by background plus analyte. High current is background absorbance only. Adapted from S.B. Smith, Jr. and G.M. Hieftje, *Appl Spectrosc*, 1983, 37, 419.

The advantages of Smith–Hieftje background correction are that the HCL serves as the single source for any analytical wavelength, obviating any alignment issues arising from alternative continuum source. This system allows correction of structured backgrounds very near the resonance wavelength. Disadvantages are that the lifetimes of the HCLs are notably shortened. Compared with continuum-source background correction, the Smith–Hieftje procedure may result in a shorter dynamic range and/or may require higher sample dilutions to reduce background absorbances.

Zeeman Effect

The Zeeman effect background correction is based on the principle that in the presence of a magnetic field, the absorption line of an element is split into three optically polarized components. The π component is positioned at the center of the resonance line. Positioned at equal distances on either side of the π component are the σ+ and σ– components. The σ components are linearly polarized perpendicular to the magnetic field. The distance of the separation of the σ+ and σ– components from the π component increases as the magnetic field strength increases. Commercial instruments use magnets with field strengths of approximately 1 Tesla, which corresponds to a σ component separation of approximately 0.01 nm on each side of the π component.

Absorbance lines from different elements have normal or anomalous Zeeman patterns. Normal Zeeman patterns are composed of a single π component and two σ components. A normal Zeeman pattern occurs for absorbance wavelengths that correspond to a singlet electron transition. An anomalous Zeeman pattern has further splitting of the π and σ components and occurs for wavelengths that correspond to more complex electron transitions.

Three basic designs are available for commercial AAS equipped with Zeeman background correction: transverse DC Zeeman AAS, transverse AC Zeeman AAS, and longitudinal AC Zeeman AAS.

TRANSVERSE DC ZEEMAN ATOMIC ABSORPTION SPECTROMETRY

This design uses a DC magnet positioned around the atomizer. The term *transverse* refers to the parallel alignment of the magnetic field with the optical axis. The Zeeman effect is permanently applied in this design. A rotating polarizer is positioned in the light path between the line source and the atomizer. This splits the incident radiation from the line source into the characteristic π and σ components. See *Figure 7* for a schematic of the configuration and an illustration of the operation of DC Zeeman AAS.

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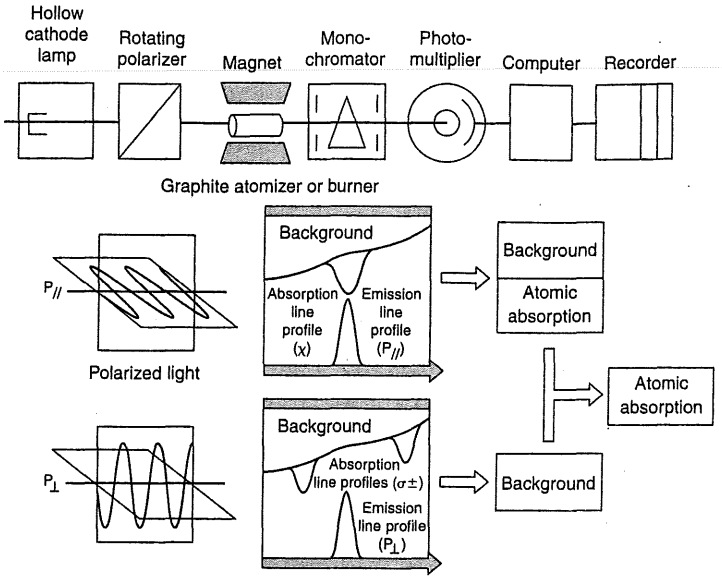

Figure 7. Configuration and operation of a transverse DC Zeeman atomic absorption spectrophotometer. Adapted from H. Koizumi and K. Yasuda, *Spectrochim Acta*, 1976, 31B, 523.

TRANSVERSE AC ZEEMAN ATOMIC ABSORPTION SPECTROMETRY

This design uses an AC magnet positioned around the atomizer. The magnetic field is in parallel alignment with the optical axis. The AC magnet is switched on and off at one-half the modulation frequency of the line source (50 or 60 Hz, typically). A fixed polarizer is positioned between the atomizer and the monochromator. See *Figure 8* and *Figure 9* for illustrations of the operation of transverse AC Zeeman AAS.

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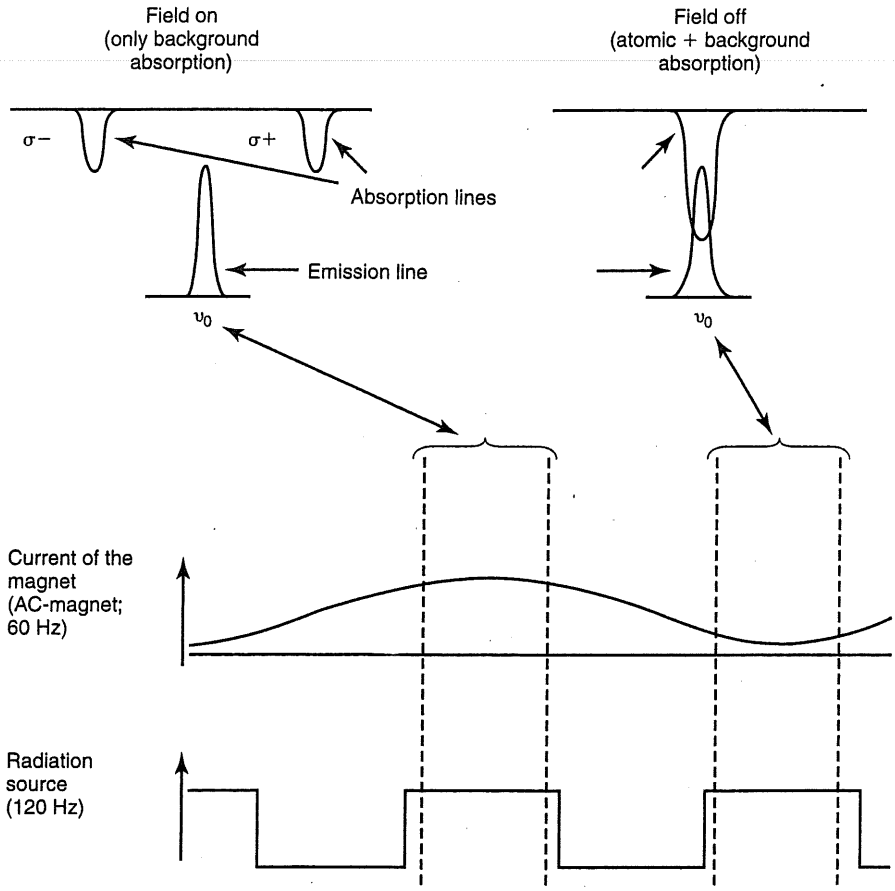

Figure 8. Operational principle of a transverse AC Zeeman atomic absorption spectrophotometer. ©2011 PerkinElmer, Inc. All rights reserved. Printed with permission.

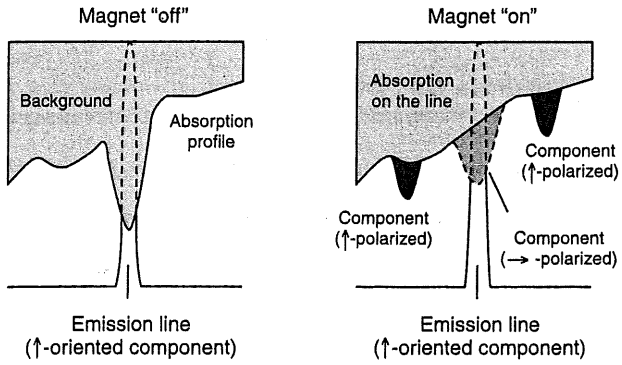

Figure 9. Illustration of transverse AC Zeeman atomic absorption spectrophotometer operation. ©2011 PerkinElmer, Inc. All rights reserved. Printed with permission.

LONGITUDINAL AC ZEEMAN ATOMIC ABSORPTION SPECTROMETRY

In this design, the magnetic field is in perpendicular alignment with the optical axis. With this configuration the π component is absent and the σ components are circularly polarized, so a polarizer is not required. See *Figure 10* for comparison between transverse and longitudinal AC Zeeman AAS.

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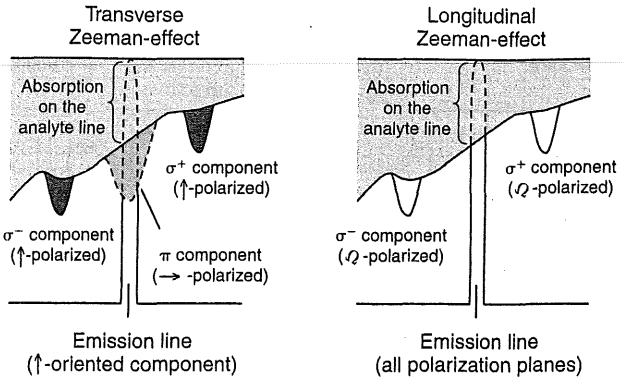

Figure 10. Comparison of transverse and longitudinal AC Zeeman atomic absorption spectrophotometer. ©2011 PerkinElmer, Inc. All rights reserved. Printed with permission.

Zeeman effect background correction offers the advantage of background correction at the resonance wavelength and correction of relatively high nonspecific absorbances. Compared to continuum source background correction, Zeeman effect background correction can result in a reduction of the linear dynamic range in some applications and may require additional dilution of test solutions. Depending on the specific analyte and application, Zeeman effect background correction offers advantages and disadvantages that must be considered for each application.

ANALYTICAL CONSIDERATIONS

Sample Preparation

FAAS requires the introduction of a liquid sample into the nebulizer. ETV/GFAAS is normally performed using liquid samples, but analyses can be performed using slurries and solid samples. Because the analysis of liquid slurries is far more common, the analysis of solid samples is not discussed in this chapter.

Samples can be prepared in a variety of ways to dissolve or solubilize them. In some cases, direct dilution of a solid sample can be performed using deionized water, dilute acids, or organic solvents. When a sample does not dissolve, however, some form of acid digestion is required. Acid digestion is also required if the sample is first ashed. Options for acid digestion include hot-plate digestions, open-vessel microwave digestion, and closed-vessel microwave digestion. Procedures for acid digestion are specific for a given sample matrix and analyte. When using microwave digestion systems, analysts should refer to the microwave manufacturer's guidelines for use and programming of the instrument.

Interferences

AAS is subject to several types of interferences. The most common are:

- Spectral interferences: Spectral interferences can arise when there is an overlapping signal from another element that is a component of the sample or sample matrix.
- Ionization of the analyte: Some elements such as sodium, potassium, calcium, and cesium are easily ionized, and ionization of the analyte reduces the analytical signal.
- Matrix effects: Interferences can arise from differences between sample, standard, and blank viscosities or can be introduced by surface tension.
- Spectral line broadening: Spectral line broadening can occur as a result of several factors, including self-absorption, Lorentz effect, Doppler effect, or quenching.
- Compounds that do not dissociate in the flame.

Matrix Modification, Releasing Agents, and Ionization Suppressants

To counteract potential interferences or to enhance their ability to monitor an analyte, analysts sometimes use a matrix modifier, a releasing agent, or an ionization suppressant.

Matrix modifiers are added to samples, standards, and blanks with GFAAS with the chief goals of changing the nature of the sample or analyte in the sample by:

- Increasing the volatility of the sample matrix so that matrix components are removed during the ashing or pyrolysis step
- Reducing the volatility of the analyte, which helps to eliminate loss of the analyte during the ashing or pyrolysis step
- Reducing background absorption by eliminating matrix components so that they do not interfere with the analyte signal during atomization.

It may be necessary to use more than one matrix modifier during an analysis. Commonly used matrix modifiers include magnesium nitrate, nickel nitrate, palladium, and lanthanum. Manufacturers of graphite furnace instrumentation provide detailed information regarding the use of matrix modifiers, and analysts should consult that information when they perform an analysis. In some instances, GFAAS analyses require applying the method of standard additions in order to overcome matrix

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induced result biases. Many modern GFAAS systems are equipped with autosamplers that automatically spike sample solutions at multiple levels specified by the analyst, perform the linear regression, and report the final concentration result based upon the intercept.

Releasing agents and ionization suppressants are used in FAAS to eliminate certain potential interferences. Releasing agents are added in excess to samples, standards, and blanks to prevent the formation of refractory compounds by combining with a potential interferent. Ionization suppressants are added in excess to samples, standards, and blanks to help control ionization of the analyte. By adding an ionization suppressant that has an ionization potential lower than that of the analyte, analysts create an excess of electrons in the flame, and the ionization of the analyte is suppressed. Commonly used releasing agents include lanthanum and strontium. Commonly used ionization suppressants include sodium, potassium, cesium, and lanthanum. Manufacturers of flame atomic absorption (AA) instrumentation provide detailed information regarding the use of releasing agents and ionization suppressants, and analysts should consult that information when performing an analysis.

Because of the separation of the analyte from the matrix in cold-vapor and hydride-generation AA, spectral interferences are notably reduced when compared to the other AA methods. Nonselective background interferences can occur, however, when a sufficient amount of an absorbing species is transported to the cell. Typically this occurs when an excess of another hydride-forming species is introduced from the sample matrix or in the case of hydride formation when nonaqueous media are used. Transport interferences take place during transport of a hydride from the sample solution to the sample cell. This either causes a delay in hydride formation, known as transport kinetics interference, or loss of the hydride altogether, known as transport efficiency interference. Volatile compounds transported to the cell with the hydride can also interfere nonselectively. These interferences can be remedied either by using the method of standard additions or by further diluting the sample.

APPENDIX

Acronyms

A: absorption
AA: atomic absorption
AAS: atomic absorption spectrometry
CVAAS: cold vapor AAS
EDL: electrodeless discharge lamp
ETV: electrothermal vaporization
FAAS: flame AAS
GFAAS: graphite furnace AAS
HCL: hollow cathode lamp
HGAAS: hydride generation AAS
PMT: photomultiplier tube
T: transmittance

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⟨1853⟩ FLUORESCENCE SPECTROSCOPY—THEORY AND PRACTICE

THEORY

Fluorescence is a two-step process that requires an initial absorption of light followed by emission. [NOTE—Many terms and variables used in this general chapter are explained in the *Glossary*.] Fluorescence spectroscopy is an electronic spectroscopic method related to ultraviolet–visible–near infrared (UV–Vis–NIR) absorption spectroscopy. It is also a background-free method that involves light emitted from the sample in all directions, as is the case with Raman spectroscopy. The initial absorption of a photon by a molecule in the sample promotes an electron to an excited state. The excited electron returns to the ground electronic state by emitting a photon. If the emission arises from an "allowed" transition that typically has a short lifetime between 1 ns and 10 ns, then it is called *fluorescence*. If the emission arises from a "forbidden" transition that typically has a long lifetime between 1 ms and 1 s, then it is called *phosphorescence*. Under similar conditions phosphorescence usually is less

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intense than fluorescence. This general chapter discusses fluorescence spectroscopy, but many points raised here also apply to phosphorescence. The basic concepts behind fluorescence spectroscopy have been well established, but its applications and standardization are still expanding and progressing, making it a developing rather than a mature method.

The most common type of fluorescent sample is a dilute, transparent solution that absorbs light following the Beer–Lambert Law and that emits a corresponding fluorescence intensity that is directly proportional to the concentration, the absorptivity, and the fluorescence quantum yield of the fluorescent species or fluorophore. A conventional fluorescence spectrometer has both excitation and emission wavelength selectors. It collects a spectrum by fixing the wavelength of one of the selectors and scanning the other wavelength selector over a range. When the excitation wavelength is fixed and the emission wavelength is scanned, the resulting spectrum is termed an *emission spectrum*. When the emission wavelength is fixed and the excitation wavelength is scanned, the resulting spectrum is termed an *excitation spectrum* (*Figure 1*). The fluorescence spectrum is plotted as relative intensity or counted photons of fluorescence vs. wavelength. The appearance of a fluorescence spectrum is much like a UV–Vis–NIR absorption spectrum. In fact, the shape or contour of an excitation spectrum often is identical to that of the corresponding absorption spectrum for an organic dye in solution over the same wavelength range.

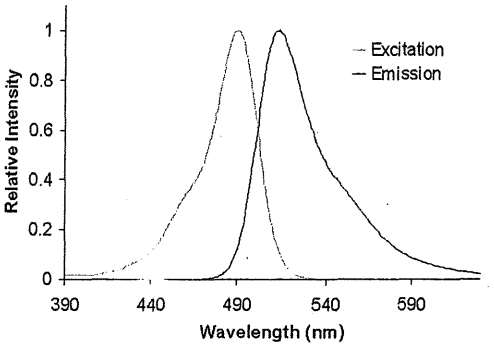

Figure 1. Fluorescence excitation and emission spectra for fluorescein in borate buffer. The wavelength axis shows excitation and emission wavelengths.

Polyatomic fluorophores in condensed media (e.g., solutions, thin films, and solids at room temperature) exist in ground or excited electronic states in a broad distribution of vibrational energy levels and cause homogeneous broadening of excitation or emission spectra, respectively. A microenvironment or shell also surrounds each fluorophore in condensed media, and differences in the structures of these shells among individual fluorophores cause inhomogeneous broadening. These two types of broadening cause fluorescence spectra to be broader than some other types of spectra (e.g., mid-infrared or Raman spectra). The typical width of a fluorescence band is between 10 nm and 100 nm. Once it is electronically excited, a polyatomic fluorophore experiences vibrational relaxation before emitting a photon, causing a red shift or Stokes shift of the fluorescence spectrum relative to the wavelength at which it was excited.

Few naturally occurring biological compounds fluoresce strongly. However, the development of a large array of fluorescent indicator dyes, used mainly to bind to ions or indicate pH or polarity, has led to increased interest in the use of both direct and secondary fluorescence techniques, e.g., fluorescence resonance energy transfer (FRET). While many of these probes inherently do not have very high quantum yields, their fluorescence changes greatly upon binding and upon the associated solution chemistry. For instance, the pH of the sample solution is an important factor to control when considering not only its impact on the fluorescence of the probe, but also on the detection of other possible fluorescing of fluorescent dye binding species. Some fluorescent probes, such as fluorescein (pH dependent) and rhodamine and its derivatives, have very large absorptivities and quantum yields approaching one—i.e., they fluoresce nearly as many photons as they absorb. Fluorescence methods are also termed *background-free* because very little excitation light reaches the detector. These advantages make fluorescence detection highly sensitive, down to single-molecule detection in some cases. Specificity and sensitivity are two of the more important strengths of fluorescence methods. Fluorescence spectroscopy also typically is not destructive to the sample, and measurements can be made quickly (on the order of seconds to minutes).

A right-angle or 0°/90° geometry often is used to measure dilute solutions and other transparent samples. In such cases, the excitation beam is normal to the sample, and fluorescence is detected at a 90° angle relative to the beam (*Figure 2a*). A front-face geometry is used to measure optically dense samples where the excitation beam is incident on the sample at <90° and the fluorescence is collected at an angle ≤90° (*Figure 2b*). The epifluorescence geometry is a special case of the front-face geometry that often is used in fluorescence microscopy and optical fiber–based fluorometers. In epifluorescence geometry the excitation beam and collected fluorescence are both normal to and are on the same side of the sample, i.e., a 0°/0° geometry. A 0°/180° transmitting geometry often is used in microscopy (*Figure 2c*).

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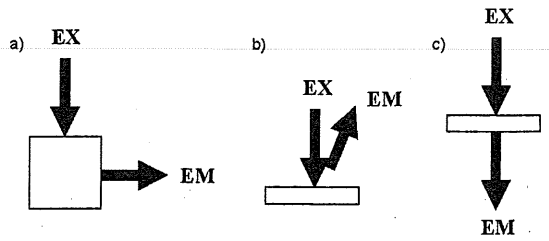

Figure 2. Schematic of the excitation beam (EX) and detected emission (EM) orientations for (a) 0°/90° right-angle transmitting, (b) front-face, and (c) 0°/180° transmitting geometries.

The number of chemical assays and screening methods using fluorescence detection continues to increase rapidly and has resulted in a corresponding increase in the need for standardization of fluorescence measurements. Only a few standard methods and reference materials have been well established and are readily available at present for the characterization of fluorescence measuring systems. National metrology institutes and international standards organizations are working to provide new fluorescence standard materials and methods in the near future. This general chapter briefly discusses the major issues that should be considered by users of fluorescence instruments who aim to achieve high-quality measurements. Standard methods and materials are also described where appropriate. A few guidelines and recommendations have appeared, but this general chapter aims to be most useful to nonexpert users of fluorescence spectrometers.

INSTRUMENTATION

All modern fluorescence measurements involve irradiating the sample with the beam from a suitable light source, selecting the excitation wavelength, collecting the resulting fluorescence, rejecting the Rayleigh-scattered light, selecting the emission wavelength, and detecting the fluorescence signal. The following functions will be discussed individually, along with the equipment used to achieve these functions in commercial instruments:

1. excitation light source
2. excitation wavelength selector
3. sampling device
4. emission wavelength selector
5. detector.

Excitation Light Source

A variety of lamps, lasers, and light-emitting diodes (LEDs) are used as excitation sources. Continuous and pulsed versions of these sources are used for steady-state and time-resolved instruments, respectively. Xenon lamps are the most commonly used because of their relatively high intensity and broad wavelength range (UV to NIR). Lasers are the highest-intensity sources and are used in applications where short collection times and small amounts of sample are required, e.g., for flow cytometry or microarrays.

Excitation Wavelength Selector

The intensity of scattered light at the excitation wavelength (i.e., Rayleigh scattering) can be comparable to or greater than that of the fluorescence at the sample. Therefore, the excitation wavelength profile should not overlap the emission wavelength region being detected. This is achieved for lamps by using an excitation wavelength selector (e.g., a filter or a monochromator with a known peak transmission wavelength and bandwidth) between the lamp and sample. The inherent bandwidth of the radiation from a laser or an LED often is narrow enough that an excitation wavelength selector is not necessary. This selector also enables fluorescence excitation spectra to be resolved.

Sampling Device

The sampling device includes all optics and other equipment needed to deliver the excitation beam to the sample, collect the emission from the sample, and hold the sample in place. Sample formats include cuvettes, microwell plates, microarrays, microscope slides, and flow systems and may be accompanied by a variety of optical delivery and collection systems, including conventional transmitting, front-face, and epifluorescence systems and fiber optic–based probes.

Emission Wavelength Selector

As with the selector for excitation, the emission wavelength selector helps to ensure that the emission wavelength region being detected does not overlap with the excitation wavelength profile. This approach enables individual fluorescence bands to be detected when multiple bands are present and allows fluorescence emission spectra to be resolved. Emission wavelength selectors also are important for the rejection of stray light. Filters, monochromators, and grating polychromators often are used for emission wavelength selection.

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Detector

For the detection of emitted light, a photomultiplier tube (PMT) or a charge-coupled device (CCD) array is placed after the emission wavelength selector. The detection of the excitation beam in order to monitor its intensity commonly is done by a quantum counter detector or a photodiode placed before the sample and to which a small fraction of the excitation beam is split off from the rest.

FACTORS THAT AFFECT QUANTITATION

Instrument-Based Factors

Measurements on a fluorescence instrument require that instrument parameters such as wavelengths, bandwidths, and detector gain be set. All of these parameters can be set with varying degrees of repeatability and accuracy, depending on the instrument used. These factors can introduce measurement uncertainty or bias that is particularly significant when measured values are compared between instruments. For instance, the measured peak positions of the emission bands of two analytes may differ between instruments because of a wavelength bias. A corresponding bias between instruments could be introduced in the results of an assay that depends on the ratio of the fluorescence intensities at the two specified emission wavelengths.

The intensity of the excitation beam can change significantly with excitation wavelength or with time because of the wavelength dependence of the intensity of the light source and the transmittance of the excitation wavelength selector or the time dependence of the light source intensity. Thus analysts should monitor the excitation beam intensity and correct the measured fluorescence intensity for these fluctuations. This monitoring can be particularly important when excitation spectra are collected because the excitation intensity often has sharp peaks and valleys when lamp sources such as a xenon (Xe) lamp are used.

The responsivity of a detection system is not linear with intensity at all intensities, so analysts should know the linear intensity range of the detection system used. The linear range for most detection systems ranges from its limit of detection up to a threshold intensity above which the responsivity becomes increasingly nonlinear with increasing intensity. Analysts should establish the linear range of the fluorescence detection system before they attempt to calibrate the responsivity of the detection system.

The responsivity of the detection system also is a result of the wavelength dependence of the transmittance of the emission wavelength selector and the responsivity of the detector. These factors can affect the shape of a measured emission spectrum.

The diffraction efficiency of gratings and the responsivity of detectors often depend on polarization. Changes to instrumental polarization settings can result in changes in the observed excitation intensity and the responsivity of the detection system. Even when polarizers are not used within the instrument, the excitation beam may be polarized by the optical system itself and may affect the responsivity of the detection system, and is instrument dependent. In addition, emission polarization effects not only can cause intensity differences but also can change spectral correction factors.

The passing of multiple wavelengths by a diffraction grating can introduce unexpected sharp peaks into a fluorescence spectrum. So that incident light is diffracted at a desired wavelength, a grating equation is used to set the angle of the grating with respect to incident light:

$$m\lambda = d(\sin\alpha + \sin\beta),\ m = 0, 1, 2, \ldots$$

m = diffraction order
d = groove spacing on the grating
α = angle of the incident wavefront relative to the grating normal
β = angle of the diffracted wavefront relative to the grating normal

The value of $m\lambda$, not λ, is fixed, where m is an integer termed the *diffraction order*. Therefore, the grating equation can be satisfied by more than one wavelength for a single grating position. For instance, if a grating in an emission monochromator is set to pass 500-nm light at first order, it also will pass 250-nm light at second order. As a result, the scattered light from a 250-nm excitation beam will be detected as a peak at an emission wavelength of 500 nm unless a suitable optical filter is inserted in the beam.

Sample-Based Factors

The fluorescence intensity of optically dense samples (e.g., absorbance A >0.05 at a path length of 1 cm) does not increase linearly with concentration because of significant absorption of the excitation beam and/or emission (reabsorption) by the sample. These inner filter effects also can greatly reduce the amount of fluorescence that reaches the detector, especially when a right-angle transmitting geometry is used. The fluorescence intensity can become strongly dependent on sample position and optical geometry. At even higher concentrations, aggregation of fluorophores often occurs, causing the shape of the fluorescence spectrum to be different from that of a dilute sample and also causing nonlinear concentration behavior.

The fluorescence intensity of a sample may decrease with time of exposure to light because of photobleaching and photodegradation. This is particularly true of most organic dyes, which are the most widely used fluorescent probes. Analysts should limit the time that such samples are exposed to light in order to obtain reproducible fluorescence intensities and in some cases even reproducible spectral shapes.

The fluorescence intensity of fluorophores is temperature dependent. Typically, the rates of fluorescence quenching processes, such as collisional quenching in solutions, increase with temperature and cause a decrease in fluorescence intensity. Temperature coefficients for fluorescence intensity for particular fluorophores can be used to correct for this temperature dependence.

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The absorbance and consequently the intensity of fluorescence from a sample depend on the orientation of the sample's absorption transition dipole with respect to the polarization of the excitation light. The polarization of fluorescence is parallel to the direction of polarization of the fluorescent species' emission transition dipole. Fluorescence polarization is parallel to the orientation of the fluorescent species' emission transition dipole. Fluorescence anisotropy (r) is used to describe the extent of polarization of emission and is defined by:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

$I_{\parallel}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented parallel to the direction of the polarized excitation

$I_{\perp}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented perpendicular to the direction of the polarized excitation

A sample whose fluorophores are oriented nonrandomly and have a rotational period that is long compared to their fluorescence lifetime will emit anisotropic fluorescence. The spectral shape and intensity of such fluorescence depend on the viewing angle and the instrument's polarization factors.

A fluorophore's fluorescence intensity and peak position, and sometimes even its spectral shape, often depend on the environment, including changes caused by the solvent used, the solution's pH, or the species to which the fluorophore is bound. For the above reasons care should be taken during the experimental design of a new methodology to consider and evaluate these effects, and matrix match the reference and unknown as appropriate.

A Raman signal can introduce peaks into the fluorescence spectrum. The Raman peaks of the sample's solvent or matrix are those most commonly encountered. For instance, the Raman peak of water, which is found red-shifted 3382 cm^{-1} from the chosen excitation wavelength, typically is observed in the fluorescence spectrum of any aqueous solution excited by UV or blue light.

CALIBRATION OF FLUORESCENCE INSTRUMENTS

Two types of fluorescence instrument calibrations are used. The first and most commonly used is analyte specific and determines the relationship between the response of the instrument (fluorescence intensity) and the concentration or amount of a specific analyte. The second is analyte independent and is intended for spectral instruments. In this case, the wavelength accuracy for emission and excitation and the spectral responsivity of the detection system are calibrated across the entire or a continuous part of the wavelength range of the instrument.

Analyte Concentration—Calibration Curves

Calibration curves of instrument response (fluorescence intensity) vs. analyte concentration are determined using reference materials that contain the analyte of interest. For instance, the fluorescence intensities of a set of solutions at different, known analyte concentrations that cover a desirable concentration range can be measured and plotted vs. concentration. The plot then is fitted to a polynomial, typically a straight line. The resulting calibration curve is both analyte and instrument specific and can be used to determine analyte concentrations of unknown samples. This type of calibration may not be accurate when the microenvironment surrounding the fluorophore is different in the reference and unknown samples. In addition, users must ensure that the fluorescence intensities of samples are reproducible and do not decrease over the time when they are being excited and measured because the organic dyes typically used may be prone to photobleaching.

In some cases, calibration samples at known concentrations and prepared from appropriate reference materials may not be available. Firstly, organic dyes that are used as fluorescent probes often are not commercially available at a known high purity that enables production of reference solutions. Secondly, in complex systems where fluorophores are bound to large molecules, cells, or microbeads, the concentration of bound fluorophores in a solution or suspension may be difficult to determine. In the latter case, the molecules of equivalent soluble fluorophore (MESF) scale has been proposed as an alternative way to use calibration curves to quantify fluorescence intensity for a particular analyte.

Emission Wavelength and Spectral Slit Width

A variety of reference standards have been proposed for use in the determination of emission wavelength accuracy, including atomic lamps and inorganic and organic fluorophores. The most widely used and best characterized of these are low-pressure atomic lamps, commonly termed *pen lamps*. In this case, the type of pen lamp (e.g., Hg, Xe, etc.) is chosen so that its radiated atomic lines are within the desired wavelength range. The lamp is placed at the sample position so that its light is centered in the optical path of the detection system of the instrument. The accuracy of this method may decrease if the pen lamp is not properly aligned. The emission wavelength selector–detector then measures the signal over the wavelength range of interest. The measured wavelength positions of the resulting sharp peaks then are compared with the known positions to determine wavelength accuracy.

The spectral slit width accuracy of the emission wavelength selector can be confirmed by measuring the spectral bandwidth, taken to be the full width at half the peak maximum, of a single line of a pen lamp. For fluorescence spectrometers with both excitation and emission monochromators, an alternative method can be used when one monochromator is scanned over the position of the other.

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Excitation Wavelength and Spectral Slit Width

Many of the reference samples that are used for determining emission wavelength accuracy also can be used for excitation wavelength accuracy. For instance, a pen lamp can be placed at the excitation light source position so that the resulting spectrum is detected after the excitation wavelength selector using the instrument's reference detector. However, in this case, a relatively weak signal may limit the number of useful atomic lines, and therefore alignment of the pen lamp is more critical in this instance than for the emission wavelength accuracy determination.

Once the accuracy of the emission wavelength selector has been determined, use a diffuse scatterer, e.g., a scattering solution or a diffuse reflector, at the sample to scatter a fraction of the excitation beam into the detection system to determine excitation wavelength accuracy. One wavelength selector is set at a fixed wavelength and the other is tuned over the same wavelength to obtain a spectrum. The wavelength bias between the two wavelength selectors is equal to the difference between the set wavelength position and the observed peak position of the collected spectrum. This method can be used at any wavelength, unlike many other methods that depend on a limited number of set excitation wavelengths determined by the reference material chosen. Methods similar to those used for spectral slit width accuracy of the emission wavelength selector also can be used to determine the spectral slit width accuracy of the excitation wavelength selector.

Linearity of the Detection System

Several approaches are available to determine the detection system's linear intensity range. They can be separated into three types, based on the tools used to vary the intensity of light that reaches the detector: (1) double aperture, (2) optical filters and/or polarizers, and (3) fluorophore concentrations. The double-aperture method is the best established and probably is the most accurate when done correctly, but it also is the most difficult to perform. A variety of methods using optical filters, polarizers, or a combination of the two have been reported. These methods require high-quality, often costly, components and some user expertise. The third method is the most popular and is easiest. It uses a set of solutions obtained by serial dilution of a fluorescent stock solution that is similar to one used for obtaining calibration curves for analyte concentration, as described earlier. In this case, analysts use solutions with low concentration (A <0.05 at 1-cm path length), but fluorophore adsorption to cuvette walls may affect measurements at very low concentrations. Users must ensure that the fluorescence intensities of samples are reproducible and do not decrease over the time that they are being excited and measured because the organic dyes typically used may be prone to photobleaching.

Signal Level (Relative Emission)

Calibration of the relative responsivity of the emission detection system with emission wavelength, also referred to as spectral correction of emission, is necessary for successful quantification when intensity ratios at different emission wavelengths are compared or when the true shape or peak maximum position of an emission spectrum must be known. Such a calibration is required because the relative spectral responsivity of a detection system can change significantly over its wavelength range (*Figure 3*). Analysts should know the degree of photometric precision required for successful quantitation. The linear range of the detection system is determined before this calibration is performed, so that appropriate steps are taken (e.g., the use of attenuators) to ensure that all intensities measured during this calibration are within the linear range. When one uses an emission polarizer, the spectral correction for emission depends on the polarizer setting.

Two methods are preferred for calibrating photometric responsivity: one (*Method A*) uses light from a calibrated source (CS), and the other (*Method B*) uses certified reference materials (CRMs). Both give results that are traceable to national metrology institutes. A calibrated tungsten white light source is used most commonly for *Method A* and covers the wavelength range from about 350 nm into the NIR. Standard reference materials from the U.S. National Institute of Standards and Technology and CRMs from the German Federal Institute for Materials Research and Testing currently are available for use in *Method B*. Corrected emission spectra of some commonly used dyes also have been reported in the literature. *Method A* is more difficult to implement than *Method B* and requires periodic recertification of the CS. A third method, *Method C*, uses a calibrated detector and a calibrated diffuse reflector. This method typically has larger uncertainties than *Method A* and *Method B* but is recommended in UV and NIR wavelength regions that are not covered by the other two methods.

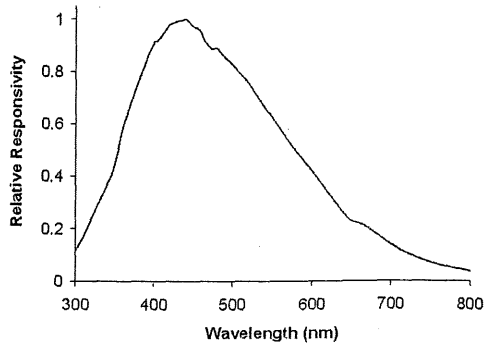

Figure 3. Example of the relative spectral responsivity of an emission detection system (PMT-based grating monochromator) for which a correction must be applied to a measured emission spectrum to obtain the true spectral shape (relative intensities).

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METHOD A

In *Method A*, analysts direct CS light into the emission detection system by placing the CS at the sample position. If the CS is too large to be placed at the sample position, analysts can place a calibrated diffuse reflector (CR) at the sample position to reflect the light from the CS into the emission detection system. The emission wavelength selector is scanned over the emission region of interest using the same instrument settings as used with the sample, and the signal channel output (S'') is collected. The known radiance of the CS incident on the detection system (L) can be used to calculate the relative correction factor (C_{CS}) so that:

$$C_{CS} = L/S''$$

C_{CS} = relative correction factor
L = radiance of the CS incident on the detection system
S'' = signal channel output

The corrected emission intensity is equal to the product of the signal output of the sample and C_{CS}.

METHOD B

In *Method B*, analysts place the fluorescence standard at the sample position. Its spectrum is collected and is compared to the certified spectrum according to the instructions given on the accompanying certificate, which yields spectral correction factors for the instrument.

METHOD C

Method C involves two steps: Step 1 uses a calibrated detector (CD) at the sample position to measure the flux of the excitation beam as a function of excitation wavelength. Step 2 uses a CR to reflect a known fraction of the flux of the excitation beam into the detection system. This is done by placing the CD at the sample position at a 45° angle, assuming a 0°/90° instrument geometry, and synchronously scanning both the excitation and emission wavelength selectors over the emission region of interest while collecting both the signal output and the reference output. This method allows analysts to calculate the relative correction factor. This method has larger uncertainties than those for *Method A* or *Method B* and typically is more difficult to implement.

Reference Signal Level (Relative Excitation)

Calibration of the excitation intensity with excitation wavelength is necessary for successful quantitation when analysts compare intensity ratios at different excitation wavelengths or when analysts must know the true shape or peak maximum position of an excitation spectrum. Such a calibration is necessary because the relative spectral flux of an excitation beam at the sample can change extensively over its wavelength range (see *Figure 4*). The neglect of excitation intensity correction factors can cause even greater errors than neglect of emission correction factors. Fortunately, many fluorescence instruments have a built-in reference detection system to monitor the intensity of the excitation beam. This monitoring usually is done using a photodiode, PMT, CCD, or a quantum counter detector to measure a fraction of the excitation beam that is split off from the rest of the beam. The collected reference signal can be used to correct the fluorescence signal for fluctuations caused by changes in the excitation beam's intensity. Reference detectors often are not calibrated with excitation wavelength, which introduces errors that can be particularly large over longer excitation wavelength ranges (e.g., >50 nm) or in a wavelength region where the excitation intensity changes rapidly with excitation wavelength (e.g., the UV range). When an excitation polarizer is used, the spectral correction for excitation intensity depends on the polarizer setting.

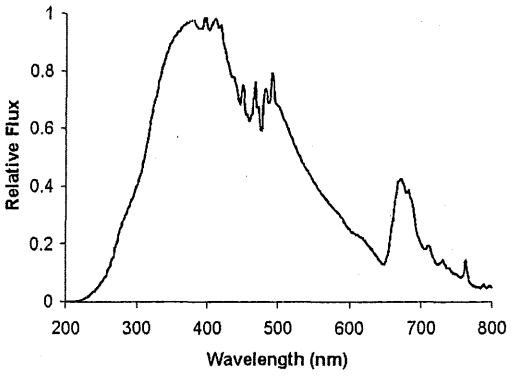

Figure 4. Example of the relative flux of an excitation beam (Xe lamp grating monochromator) for which a correction must be applied to a measured excitation spectrum in order to obtain its true spectral shape.

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When a reference detector is not built into an instrument, a spectral correction for the reference channel or an independent spectral correction of excitation intensity is required. Three methods can be used to determine the spectral correction of excitation intensity: a CD (Method 1); a calibrated diffuse reflector (Method 2); or a quantum counter (Method 3). The latter two methods use the instrument's fluorescence detection system as a detector.

For Methods 1 and 2, the detector and diffuse reflector are calibrated for responsivity and reflectance, respectively, as a function of wavelength. For Method 2, excitation and emission wavelength selectors are scanned synchronously, and the spectral correction for the emission channel [see *Signal Level* (*Relative Emission*)] must be applied to the measured intensities. Method 3 should be used only in the quantum counter's effective wavelength range where a wavelength-independent response can be achieved. Method 1 using a CD has fewer caveats than do the other two methods. A CD is placed in the sample position, and the output is measured as a function of emission wavelength by scanning the excitation wavelength selector over the excitation region of interest using the same instrument settings as those used with the sample. The known responsivity of the CD is used to calculate the flux of the excitation beam. If the instrument's reference detector is used to measure the intensity of the excitation beam simultaneously with the CD, then the correction factor for the responsivity of the reference detector also can be calculated.

Intensity and Sensitivity

The absolute value of the fluorescence signal measured by the detection system depends not only on the sample itself but also on the excitation intensity of the sample and the optical geometry of the instrument. Therefore, determination of instrument-independent fluorescence intensity of any sample or the absolute responsivity of any detection system in terms of the intensity of the sample or measured by the detector, respectively, relative to the excitation intensity can be difficult.

The most accurate way to calibrate an instrument for absolute intensity is to use conventional standards-based methods such as those that employ a calibrated light source or a calibrated detector in combination with a calibrated reflector. These methods require user skill and knowledge. Also, the (typically annual) certification and recertification are expensive. In addition, these standards tend to be bulky and are not compatible with many instruments. Thus, most researchers use simpler alternative standards and methods.

One approach is to correlate fluorescence signals to analyte concentrations using calibration curves or MESF units (see *Analyte Concentration—Calibration Curves*). Another approach is to measure the intensity of a standard sample that can be expected to always give the same fluorescence intensity under the same conditions.

Organic dyes, such as those used as fluorescent probes, generally are not good choices for intensity standards because of issues with photobleaching, stability, and reproducible concentration. If organic dyes are used, then those with known high purity and known shelf life, such as those produced by national metrology institutes, are recommended for single use (i.e., analysts should use a fresh solution for every measurement).

A better alternative is to use fluorescent samples that are stable over time even when exposed to light. For example, fluorescent, inorganic glasses with well-characterized photostability and spectral properties and long shelf lives are commercially available. Such materials can be used for determining a quasi-absolute intensity scale by measuring fluorescent signal at fixed wavelength values within their recommended range using specified experimental parameters such as bandwidths, excitation intensities, and temperatures.

The sensitivity of a fluorescence instrument is determined by measuring the signal-to-noise ratio of the fluorescence signal of intensity standards. The Raman line of water often is used to measure sensitivity in a similar way, but the Raman signal typically is strong enough only to be useful in the UV region. Analysts can use organic dye solutions to measure instrument sensitivity or limits of detection with caveats that are identical to those that apply when the solutions are used as intensity standards.

The methods outlined here yield a quasi-absolute intensity scale that should be instrument independent for instruments with similar optical geometries, designs, and settings. Results of these measurements enable comparison of the sensitivity of different fluorescence instruments, but these comparisons should be approached with caution because of the relatively large and difficult-to-quantify uncertainties involved.

PROCEDURE VALIDATION

Validation of an analytical procedure using fluorescence demonstrates that the result is valid within a specified, acceptable uncertainty budget. Instrument qualification, which also may involve instrument calibration, usually is part of the process, and analysts also must consider sample-related errors (see *Sample-based Factors*). These can arise from concentration, anisotropy, photostability, and shape of the sample, in combination with effects of the instrument's optical geometry. All suspected errors should be quantified and combined to give a total estimated error that must be less than the method-specific, acceptable limit.

GLOSSARY

Absorptivity (a): A measure of the absorption of radiation from an incident beam as it traverses a sample, which is equal to the quotient of:

$$A/bc$$

A = absorbance
b = path length (cm)
c = concentration (mg/mL)

Also referred to as *specific absorption coefficient* by the International Union of Pure and Applied Chemistry.

Absorption coefficient (α): A measure of absorption of radiation from an incident beam as it traverses a sample according to Bouguer's Law:

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$$I/I_0 = e^{-ab}$$

I = transmitted intensity
I_0 = incident intensity
e = base of natural logarithm
a = absorptivity
b = path length of the beam through the sample

Note that transmittance $T = I/I_0$ and absorbance $A = -\log T$.

Beer–Lambert law (or Beer's law or Beer–Lambert–Bouguer law): In the absence of any other physical or chemical factors, A_λ is proportional to path length, b, through which the radiation passes and to the concentration, c, of the substance in solution in accordance with:

$$A_\lambda = \varepsilon_\lambda cb$$

ε_λ = molar absorptivity
c = solute concentration (M/L)
b = path length (cm)

Calibrated detector (CD): A light detector whose responsivity as a function of wavelength has been determined along with corresponding uncertainties.

Calibrated light source (CS): A light source whose radiance as a function of wavelength has been determined along with corresponding uncertainties.

Calibrated diffuse reflector (CR): A Lambertian reflector whose reflectance as a function of wavelength has been determined along with corresponding uncertainties.

Certified reference material (CRM): A material with properties of interest, the values and corresponding uncertainties of which have been certified by a standardizing group or organization. A "reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures" [International Vocabulary of Metrology (VIM) 5.14].

Diffuse scatterer: A material that scatters light in multiple directions. This includes diffuse reflectors, which often are Lambertian, and scattering solutions, which are not Lambertian.

Fluorescence anisotropy (r): A measure of the degree of polarization of fluorescence:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

$I_{\parallel}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented parallel to the direction of the polarized excitation
$I_{\perp}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented perpendicular to the direction of the polarized excitation

Fluorescence band: A region of a fluorescence spectrum where the intensity passes through a maximum, usually corresponding to a discrete electron transition.

Fluorescence lifetime:[1] A parameter describing the time decay of the fluorescence intensity of a sample component. If a sample decays by first-order kinetics, this is the time required for its fluorescence intensity and corresponding excited-state population to decrease to $1/e$ of its initial value.

Fluorescence quantum efficiency: The ratio of the number of fluorescence photons leaving an emitter vs. the number of photons absorbed.

Fluorescence quantum yield (Φ): The probability that a molecule or species will fluoresce once it has absorbed a photon. This quantity is an innate property of the species and typically is calculated for a sample as the ratio of the number of molecules that fluoresce vs. the number of molecules that absorb. Fluorescence quantum yield values range from 0 (i.e., no molecules fluoresce) to 1 (theoretical maximum in which all molecules that had absorbed radiation fluoresce).

Flux (or radiant flux): The rate of propagation of radiant energy, typically expressed in watts. *Spectral flux* is the flux per unit spectral bandwidth, typically expressed in watts per nanometer.

Grating equation: It describes the relationship between the angle of diffraction and the wavelength of radiation that is incident on a grating:

$$m\lambda = d(\sin\alpha + \sin\beta)$$

m = diffraction order
d = groove spacing on the grating
α = angle of the incident wavefront relative to the grating normal
β = angle of the diffracted wavefront relative to the grating normal

Inner filter effects: A decrease in the measured quantum efficiency of a sample caused by extensive absorption of the excitation beam or reabsorption of the emission by the sample itself. This causes the measured quantum efficiency to depend on the absorbance, concentration, and excitation and emission path lengths of the sample.

Intensity: A measure of the amount of electromagnetic energy present. This general definition is synonymous with or directly proportional to the signal output of a photodetector or the flux of a sample or light source. A more specific definition

[1] Boens N, Qin W, Basaric N, et al., Fluorescence lifetime standards for time and frequency domain fluorescence spectroscopy. *Anal Chem.* 2007;79(5): 2137–2149.

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often used in radiometry is: the radiant flux per unit solid angle from a point source, which typically is expressed as watts per steradian (*W/sr*). [NOTE—Steradian corresponds to the SI unit of solid angle.]

Lambertian reflector: A surface that reflects light according to Lambert's law, i.e., the light is unpolarized and has a radiance that is isotropic or independent of viewing angle.

Limit of detection (LOD): An estimate of the lowest concentration of an analyte that can be measured with a given procedure, often taken to be the analyte concentration with a measured signal-to-noise ratio of 3.

Noise level: The peak-to-peak noise of a blank.

Photobleaching: A loss of emission or absorption intensity by a sample caused by exposure to light. This loss can be reversible or irreversible, and the latter typically is referred to as *photodegradation* or *photodecomposition.*

Quantum counter: A photoluminescent emitter with a quantum efficiency that is independent of excitation wavelength over a defined spectral range. When a quantum counter is combined with a detector to give a response proportional to the number of incident photons, the pair is called a *quantum counter detector.*

Quasi-absolute fluorescence intensity scale: A fluorescence intensity scale that has been normalized to the intensity of a fluorescent reference sample or artifact under a fixed set of instrumental and experimental conditions. This artifact should demonstrably yield a fluorescence intensity that is reproducible with time and between instruments under a fixed set of conditions.

Raman scattering: The inelastic scattering of radiation (the wavelengths of the scattered and incident radiation are not equal) by a sample that occurs because of changes in the polarizability of the relevant bonds of a sample during a molecular vibration. Unlike fluorescence, the radiation being scattered is not required to be in resonance with electronic transitions in the sample.

Rayleigh scattering: The elastic scattering of radiation by a sample; i.e., the scattered radiation has the same energy (same wavelength) as the incident radiation.

Responsivity (spectral): The ratio of the photocurrent output and the radiant power collected by a light-detection system. *Spectral responsivity* is the responsivity per unit spectral bandwidth.

Sensitivity: A measure of an instrument's ability to detect an analyte under a particular set of conditions.

Spectral bandwidth (or spectral bandpass or resolution): A measure of the capability of a spectrometer to separate radiation or resolve spectral peaks of similar wavelengths. Usually observed as the triangular dispersion of an emission line, this parameter is taken to be the full width at half the peak maximum (FWHM).

Spectral slit width: The mechanical width of the exit slit of a spectrometer divided by the linear dispersion in the exit slit plane. In practice, observed as the triangular dispersion of an emission line, this width includes all the transmitted wavelengths for a given slit setting.

Transition dipole moment: An oscillating dipole moment induced in a molecular species by an electromagnetic wave that is resonant with an energy transition of the species, e.g., an electronic transition. Its direction defines the transition polarization, and its square determines the intensity of the transition.

⟨1854⟩ MID-INFRARED SPECTROSCOPY—THEORY AND PRACTICE

PRINCIPLES OF MID-INFRARED SPECTROSCOPY

Mid-infrared (mid-IR) spectroscopy involves measurement of the absorption of electromagnetic radiation over the wavenumber range of 4000–400 cm^{-1} (which corresponds to the wavelength range of 2.5–25 μm) caused by the promotion of molecules from the ground state of their vibrational modes to an excited vibrational state. The most commonly used parameter to denote the energy of the transitions is the wavenumber, i.e., the number of waves per centimeter. The wavelength, λ (μm), and wavenumber, $\tilde{\nu}$ (cm^{-1}), of radiation are related by the expression:

$$\tilde{\nu} = 10^4/\lambda \qquad (1)$$

The mid-IR spectrum extends from 4000 cm^{-1} (2.5 μm) to 400 cm^{-1} (25 μm). Molecules can move in a certain number of vibrational modes. The energy of mode, *i*, is given by:

$$E_i = hc\tilde{\nu}_i\left(v_i + \tfrac{1}{2}\right) + hc\tilde{\nu}_i \times x_i\left(v_i + \tfrac{1}{2}\right)^2 \qquad (2)$$

h = Planck's constant
c = velocity of light
$\tilde{\nu}_i$ = fundamental vibrational frequency of mode i (cm^{-1})
v_i = vibrational quantum number of this mode
x_i = so-called anharmonicity constant

The strongest bands in the mid-IR spectrum are caused by fundamental transitions from the ground state of a given mode ($v_i = 0$) to its first excited vibrational state ($v_i = 1$), although weaker overtone and combination bands also are observed in the spectrum. Overtone bands are caused by the promotion of molecules from their ground state to their second and higher vibrational states ($v_i = 2$, 3, etc.). Overtones are observed only for those modes for which x_i is non-zero. Combination bands are caused by the simultaneous promotion of molecules to two excited vibrational states.

Vibrational modes involve the motion of all atoms of the molecule. Many modes involve only large-amplitude vibrations of the atoms in localized regions of the molecule, and the remaining atoms are largely unaffected. When molecules contain a certain functional group, the transitions often occur in narrow spectral ranges. In this case, the wavenumbers at which these

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transitions occur are known as *group frequencies*. When a vibrational mode involves atomic motions of more than just a few atoms, the frequencies occur over wider spectral ranges and are not characteristic of a particular functional group. Instead, they are more characteristic of the molecules as a whole. Such bands are known as *fingerprint bands*. All strong bands that absorb at wavenumbers above 1500 cm^{-1} are group frequencies. Strong bands that absorb below 1500 cm^{-1} can either be group frequencies or fingerprint bands. Thus, not all strong bands in the IR spectrum of a given molecule can be attributed to the presence of a particular functional group.

The motion of atoms during a particular vibrational mode, *i*, is characterized by the normal coordinate, Q_i. The intensity of fundamental bands is governed by the square of the change in dipole moment, μ, during the vibrational cycle $(\partial\mu/\partial Q_i)^2$. Thus vibrational transitions of modes involving polar groups, such as C–O, C=O, O–H, N–H, and C–F, typically give rise to strong bands in the spectrum. Where $(\partial\mu/\partial Q_i)$ is small, transitions are weak. When the symmetry of a molecule leads to the condition that $(\partial\mu/\partial Q_i) = 0$ for a certain mode, the band corresponding to this mode does not appear in the IR spectrum. Overtone and combination bands are always weaker than the fundamental modes from which they are derived.

For functional groups that have the form XY_2 (such as $-CH_2$, $-NH_2$, $-NO_2$, and $-SO_2$) and XY_3 (such as $-CH_3$ and $-NH_3^+$), first-order coupling can occur so that the mode is split into a symmetrical mode (where both the Y atoms move to and from the X atom with the same phase) and an antisymmetrical mode (where one Y atom moves 180° out of phase with the other). The amount by which the two bands are split depends on the Y–X–Y angle. The more closely this angle approaches 180°, the greater the splitting. Thus, for example, for the symmetric and antisymmetric stretching modes of ketenes and isocyanates where the angle is approximately 180°, the splitting can approach 1000 cm^{-1}, whereas for CH_2 and CH_3 groups where the angle is approximately 108°, the splitting is on the order of 100 cm^{-1}.

Second-order coupling, sometimes known as Fermi resonance, occurs when an overtone or combination band happens to occur at the same (or near coincident) wavenumber as a fundamental mode of the molecule that involves motion of the same atoms. In this case, the overtone or combination borrows intensity from the fundamental band, and the two bands split apart by as much as 40 cm^{-1}. The closer the bands that interact in this manner, the greater the splitting and the closer the intensity for the resulting bands.

SAMPLING PROCEDURES

Mid-IR spectra can be measured by transmission, external reflection, internal reflection [often called attenuated total reflection (ATR)], diffuse reflection, and photoacoustic spectroscopy. Each of the major approaches is presented below.

Transmission Measurements

The alkali halide disk and mulling procedures are the traditional mid-IR transmission sample presentation methods for materials that are in the form of a finely divided powder, as is the case for many drug substances and excipients. During preparation of a sample suitable for IR spectroscopy, the powdered material is uniformly dispersed throughout either the alkali halide or mulling agent, which acts as a support matrix for the analyte. Other procedures by which transmission spectra can be acquired include the use of solutions and compression cells. Neat compounds can be examined in a compression cell, as a self-supporting film (for polymers), as a capillary film between the IR-transparent cell windows (for liquids and semisolids), and as a gas.

The ratio of the single-beam spectrum of the sample and an appropriate background spectrum at a given wavenumber, $\tilde{\nu}$, is known as the transmittance, $T\text{-}(\tilde{\nu})$. A transmittance spectrum is the direct output of most prism or grating spectrophotometers. For spectra measured on a Fourier transform-IR (FT-IR) spectrophotometer, the two single-beam spectra are measured at different times and are ratioed subsequently. An appropriate background spectrum should be measured. For alkali halide disks and mineral oil mulls where the diameter of the sample is usually greater than that of the focused beam, the background is measured with nothing in the sample compartment. If the sample diameter is smaller than that of the beam, the empty sample holder should be in place when the background spectrum is being measured. Similarly, the background for measurements made with an IR microscope should be measured with the same aperture that is used to measure the sample spectrum. For the measurement of ATR spectra, the background spectrum should be the clean internal reflection element.

Because transmission spectra of nonscattering samples obey the Beer–Lambert Law (usually abbreviated as Beer's Law), the transmittance commonly is converted to absorbance, $A\ (\tilde{\nu})$, i.e., $\log_{10} 1/T\ (\tilde{\nu})$. Beer's Law states that the absorbance of component *i* at wavenumber, $A_i\ (\tilde{\nu})$, is the product of the absorptivity of *i* at that wavenumber, $a_i\ (\tilde{\nu})$, the path length of the sample, *b*, and the concentration of *i*, c_i. The measured absorbance of a mixture at each wavenumber is the sum of the absorbances of each component of the mixture. Certain powdered alkali halides, such as potassium bromide, potassium chloride, and caesium iodide, coalesce under high pressure and can be formed into self-supporting disks that are transparent to mid-IR radiation.

Potassium Bromide (KBr) Disks

The alkali halide most commonly used is powdered, dry, highly pure potassium bromide, which is transparent to mid-IR radiation to approximately 400 cm^{-1}. From this point, alkali halide disks will be referred to as *KBr disks* even though they can be made with other alkali halides such as potassium chloride and caesium iodide. IR spectroscopic-grade potassium bromide with a particle size of 100–200 mesh (about 100 μm in diameter) can be purchased commercially. Nonspectroscopic-grade material may contain impurities with absorption bands in the mid-IR region. One of the more common impurities is potassium nitrate, which has a sharp absorption band at approximately 1378 cm^{-1}. Powdered potassium bromide has a tendency to adsorb molecules from the air over a long period of time, so it must be stored properly. If the potassium bromide is not dry, its spectrum exhibits a broad absorption band caused by adsorbed water at approximately 3400 cm^{-1}, along with a weaker band near 1640 cm^{-1}.

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Commercial presses and dies in a range of diameters are available for the preparation of alkali halide and similar disks. The most common diameter of KBr disks is 13 mm, but mini-disks with a diameter as small as 0.5 mm can be prepared using commercially available presses. Follow the manufacturer's recommended procedures for operating the disk-making accessory and the press.

Typically, the weight ratio of sample to alkali halide is on the order of 1 part of the sample to 100–400 parts of potassium bromide. An optimal procedure for preparing a 13-mm diameter disk is to pregrind the sample to a fine particle size in an agate mortar. A vibrating agate or steel ball mill also can be used. Then 1–2 mg of the ground sample is weighed and transferred to a clean mortar (or vial). A weighed amount of dry ground potassium bromide powder (300 mg) is then added and mixed gently with the analyte to form a homogeneous mixture. The optimum way of achieving homogeneity is to add about 10 mg of ground potassium bromide to the sample in a mortar and then to gently mix with the pestle. This is followed by successively adding doubled quantities of potassium bromide (i.e., approximately 20, 40, 80, and 160 mg) with mixing after each addition. The applied pestle action should induce good mixing with minimal grinding because further reduction of the potassium bromide particle size will lead to increased water absorption. (If the mixing is done in a vial, then the mixing time is shortened.) The mixture of potassium bromide and analyte is then transferred completely to a clean 13-mm die, which is filled and assembled according to the manufacturer's instructions. When the die is connected to a rotary vacuum pump, evacuate for about 2 min. The die (still under vacuum) is placed in a hydraulic press, and a pressure sufficient to form a disk that shows uniform transparency is applied. Discard any disk that visually shows lack of uniform transparency or exhibits poor transmittance at about 2000 cm^{-1}. Faulty, unsatisfactory, or poor-quality disks may be a consequence of inadequate or excessive grinding, moisture/humidity, or impurities in the dispersion medium.

Mulls

To prepare a mull, homogeneously distribute the finely divided powder sample in a thin layer of a viscous liquid that is semi-transparent to mid-IR radiation and has a refractive index closely matched to that of the sample. The prepared mull is sandwiched between a pair of mid-IR–transparent windows, and a transmission spectrum of the mull preparation is recorded. The mull, which should have the consistency of a paste, is formed in such a manner as to minimize radiation scattering effects (radiation scatter from particles is worse when there is greater mismatch between the refractive index of the dispersant and surrounding medium). The sandwich can be clamped together in a mull cell. Commercial mull cells are available for both macro- and micro-preparations.

The most widely used mulling agent for the mid-IR region is a saturated hydrocarbon mineral oil (liquid paraffin, Nujol). This material has strong absorption bands of 3000–2800 cm^{-1} and of 1500–1340 cm^{-1}, and a weaker band at 720 cm^{-1} that may obscure the absorption bands of the sample in these regions. In this case, a mull may require preparation in a chemically different oil. This can be achieved by the use of a perhalogenated oil mulling agent such as chlorofluoro substituted polymers. Combining the spectra of the sample prepared in the two mulling oils and ignoring the regions where one of them has strong absorption bands enables observation of the full mid-IR absorption spectrum with minimal interference.

For the preparation of a mineral oil mull, the particle size of the sample must be reduced to below that of the shortest wavelength of the interrogating radiation (2.5 µm) in order to minimize light-scattering effects that decrease spectral contrast and cause band distortions. The spectrum from a coarse powder or one that is poorly ground will show a high degree of scatter that is manifested as a sloping baseline that decreases toward shorter wavelengths (higher wavenumbers). Also, a coarser powder will increase the Christiansen effect, which is caused by reflection from the interface between materials of different refractive index. The refractive index of materials with strong absorption bands varies in a way that is similar to the first derivative of the profile of the absorption band—a phenomenon that is referred to as *anomalous dispersion*. The Christiansen effect is manifested as a transmission increase on the short-wavelength (high-wavenumber) side of an absorption band with a concomitant decrease on the longer wavelength side. Furthermore, a coarse, poorly dispersed powder can lead to a severely distorted mid-IR spectrum in which the relative intensity of the weaker bands is enhanced and the intensity of the more intense bands in the spectrum appears weaker and distorted. These effects are a consequence of radiation that has reached the detector but has not been transmitted through a representative sample of the analyte. Similar effects can be seen in the spectra of poorly prepared KBr disks.

During the preparation of mulls and KBr disks, some work is done on the analyte, either in the form of grinding, mixing, or pressing, and consequently there is the potential to induce solid-state form transformations. Although laboratory mechanical mills can produce powders with a small particle size, hand grinding using a mortar and pestle usually achieves better control and less aggressive processing for organic pharmaceutical materials. Practitioners generally accept that of the two procedures, the mull procedure is the less aggressive and is less prone to induce solid-state form changes such as changes in the crystallinity (polymorphism) or changes in the hydration or solvation state (pseudopolymorphism). The KBr disk procedure does, however, have advantages over the mull presentation method because potassium bromide exhibits no absorption bands above 400 cm^{-1} (neglecting any adsorbed water or impurities) and is better adapted to micro-sample preparations. When the sample is a salt, as is frequently the case for active pharmaceutical ingredients (APIs), ion exchange can occur between the analyte and alkali halide, and the sample is better prepared as a mull.

Compression Cells

The use of a compression cell has become a popular sampling procedure for recording a mid-IR transmission spectrum of a small or limited-quantity solid sample such as a single particle of an API or excipient, a contaminant such as a short length of fiber, or a small fragment from a packaging material. This is particularly the case for investigations using an IR microscope system. Type IIa diamonds are quite transparent over much of the mid-IR region, although they exhibit fairly strong absorption between approximately 2000 and 2400 cm^{-1}. Because of the high strength of diamond, it is commonly used as the window material of compression cells. The sample is placed between the diamond windows of the cell, the cell is then tightened, and the sample thickness is reduced to an optimum for a transmission measurement. The compressed sample can be examined while it is contained within the compression cell.

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Self-Supported Polymer Films

The mid-IR transmission spectrum of many polymers used as packaging materials can be recorded from samples prepared as thin self-supporting films. Films of appropriate thickness can be prepared by, for example, hot compression moulding a sample or microtoming a thin section from a sample. Soft and low-melting solids that do not crystallize when cooled can be prepared either as a thin layer sandwiched between two mid-IR–transparent windows by gently warming the sample or from the melt. Thin films from some materials can be cast from solution onto an IR-transparent window.

Capillary Films

Nonvolatile liquids can be examined neat in the form of a thin layer sandwiched between two matching windows that are transparent to IR radiation. The liquid layer must be free of bubbles and must completely cover the diameter of the IR beam focused onto the sample.

Liquids and Solutions in Transmission Cells

For the examination of liquid and solution samples, transmission cell assemblies that comprise a pair of windows constructed of mid-IR–transparent materials such as potassium bromide spacers, filling ports, and a holder are available commercially in both macro- and micro-sample configurations. They can be sealed, semi-permanent, or flow-through. A wide range of standard thickness spacers and window materials is available. For laboratory applications, spacers are typically formed from lead, poly(tetrafluoroethylene), or poly(ethylene terephthalate) and can be supplied, depending on spacer materials, in standard thickness path lengths from approximately 6 µm to 1 mm or larger.

The optimum path length required for examining a particular liquid or solution usually must be determined empirically and depends on its absorption characteristics and whether the application is qualitative or quantitative. In some instances for qualitative work, transmission cells can be replaced with disposable porous media such as polyethylene or poly(tetrafluoroethylene) mounted on a suitable backing.

Gases

Mid-IR transmission cells for static or flow-through gas and vapor sampling are available in a wide range of materials to suit the application, from laboratory to process scale. In the laboratory, the traditional gas cell has been a 10-cm long cylinder made from borosilicate glass or stainless steel with an aperture of about 40 mm at each end. Each open end is covered with an end cap that contains one of a pair of mid-IR–transparent windows constructed from, for example, potassium bromide, zinc selenide, or calcium fluoride. The cell body is fitted with appropriate inlet and outlet ports. Different gases may present different sealing requirements that should be taken into account. For the detection of gases at the ppm level, long-path length cells constructed from borosilicate glass or metal are used. These cells can be of fixed path length of up to about 2 m or of variable path lengths of 10–200 m. Gas cells can be jacketed and operated at temperatures of 250° or greater, and their pressure ratings can range from vacuum to more than 50 atmospheres. Small differences in temperature and pressure have significant effects on the spectrum, and care must be taken to ensure that the calibration and analysis are performed under similar conditions such that the calibration remains valid.

Attenuated Total Reflection Spectroscopy

Attenuated total reflection (ATR) spectroscopy, alternatively known as internal reflection spectroscopy or evanescent wave spectroscopy, has become a widely used procedure. This is largely a consequence of a new generation of simple-to-use single-reflection accessories.

ATR spectroscopy relies on the optical property that radiation passing through a medium of high refractive index, n_2 [the *optically dense* medium, also known as the internal reflection element (IRE)], at an angle of incidence greater than the critical angle will be totally internally reflected at a boundary in contact with a material of lower refractive index, the sample, n_1 (the *optically rare* medium). The critical angle, θ_C, is given by n_1/n_2. The electric field of the radiation penetrates a short distance into the optically rare medium. The intensity of this electric field, which is known as the *evanescent wave*, is confined within the vicinity of the surface of the denser medium. Its intensity decreases exponentially with distance, normal to the surface, into the optically rare medium. It can, therefore, be envisaged as penetrating the surface layer of the rarer medium. The depth of penetration, d_p, is a convenient comparative term for different experimental arrangements. It is the distance from the surface of the IRE at which the amplitude of electric field amplitude falls to 37% ($1/e$) of its value at the surface:

$$d_p = \frac{\lambda_0}{2\pi n_2 \sqrt{(\sin^2\theta - n_{12}^2)}} \quad (3)$$

λ_0 = wavelength of the radiation in vacuum

θ = angle at which the beam strikes the internal surface of the IRE

$n_{12} = n_2/n_1$

Note that, to achieve total internal reflection, θ must be greater than θ_C, so that n_2 is usually greater than 2.3. Some of the more commonly used IRE materials are the following: zinc selenide ($n_2 \sim 2.4$), Type IIa diamond ($n_2 \sim 2.4$), silicon ($n_2 \sim 3.4$), and germanium ($n_2 \sim 4.0$). Furthermore, d_p decreases with increasing angle of incidence and increases with increasing wavelength (decreasing wavenumber). As a consequence of the increase in d_p with increasing wavelength, the band intensities within an

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ATR spectrum appear—by comparison with a conventional transmission spectrum—to be relatively increasingly enhanced with decreasing wavenumber.

Furthermore, the refractive index of all molecules is not constant and varies across absorption bands (anomalous dispersion). For strong absorption bands, n_1 can vary between approximately 1 and 2. This effect causes a shift in the measured wavenumber of a band with respect to transmission spectra. Particularly when equipment is operating at high angles of incidence or with an IRE with a low refractive index such as zinc selenide or diamond, strong bands may also be accompanied by the appearance of an underlying first-derivative-like shape (see above for a discussion of the Christiansen effect in KBr disks).

Many different geometrical shapes and sizes are used for IREs. A trapezoidal IRE is commonly incorporated into so-called horizontal ATR (H-ATR) units. Hemispherical IREs are the core of some micro-sampling ATR accessories. Rod or rod-like multiple internal reflection IREs are often used for on-line monitoring of liquid processes.

Multiple internal reflection (MIR) elements allow the internally reflected radiation in the IRE to interact several or many times with the surface layer of the sample with which it is in contact, thereby increasing the intensity (effective path length) of the recorded sample spectrum. Still, the spectrum recorded is characteristic only of the depth probed by a single reflection. The number of internal reflections depends on the length and thickness of the IRE. MIR elements may be several centimeters in length. A typical configuration for a vertically mounted 45° angle of incidence IRE may allow 25 internal reflections. Today the most commonly used MIR systems in the pharmaceutical laboratory are those in which the IRE is mounted horizontally. These are often referred to as H-ATR accessories. MIR systems based on cylindrical rods with cone-shaped ends or similar geometries are often incorporated as liquid-sampling devices in flow-through solution cells or are used for on-line process monitoring. The trapezoidal-shaped MIR elements incorporated into H-ATR accessories enable or facilitate study of a wide range of sample forms, including liquids, solutions, dispersions, creams, pastes, waxes, semi-solids and soft powders, continuous flat surface solids, solutions, films cast from solution, and many more. Micro– and macro–H-ATR accessories are commercially available with 1, 3, 9, or more sample-interaction reflections.

Single-reflection, simple prism, and novel design IREs are also used in commercial H-ATR units. They provide an effective and convenient means of analyzing and studying samples in a diverse range of physical forms. In particular, small contact area, single-reflection, fixed angle of incidence H-ATR accessories have become popular no-preparation sampling devices within the pharmaceutical industry because they provide a ready means to record, in a simple, quick manner, a mid-IR spectrum from a limited quantity of almost any condensed-phase material. Hemispherical IREs act as focusing lenses so that the area sampled is generally smaller than that sampled with prismatic IREs. Even though the IRE may be opaque to visible light, many accessories listed above allow some form of viewing capability so that the sample under test can be inspected.

Single-reflection micro-ATR units have the advantage of virtually no requirement for a solid sample to have a uniformly flat surface. The test sample is placed in contact with the IRE sampling area and, if it is a solid, a clamp is used to compress and secure the sample against the IRE. Hemispherical ATR elements of zinc selenide, germanium, and silicon also form the sensing elements for ATR objectives that can be fitted to mid-IR FT-IR microscopes. Several of the accessories are capable of operation at controlled elevated temperatures that permit, for instance, studies relating to thermally induced, solid-state form transformations.

The hardness, scratch resistance, chemical inertness, and mid-IR transparency over a wide wavenumber range make the Type IIa diamond a unique material for ATR measurements. Even though it does have a broad absorption feature between approximately 2400 and 2000 cm^{-1}, for most pharmaceutical applications, this is not prohibitive because this is the region in which only characteristic stretching bands occur for triple and cumulated double bonds. Because of cost, the use of Type IIa diamond as an IRE material is usually restricted to micro-ATR accessories or when the IRE is used as the sensing element in an ATR immersion probe for process monitoring. Diamond has a refractive index that is closely matched to that of zinc selenide, so composite IREs can be constructed. Lower-cost focusing or support optics made from zinc selenide can be optically interfaced with a Type IIa diamond ATR sensing element, thereby minimizing the overall cost of the IRE while still benefiting from the properties of the diamond. On the basis of this technology, 3- and 9-reflection MIR configurations have been designed for both laboratory systems and process probes.

External Reflection Spectroscopy

Several types of external reflection IR spectra can be measured. Among them are Fresnel reflection, transflection, reflection–absorption spectroscopy, and photoacoustic spectroscopy, but with the exception of diffuse reflection they are not widely used in pharmaceutical applications.

Diffuse Reflection

Spectra recorded from powders or fairly fine granular samples are known as *diffuse reflection* (DR) spectra. Most of the spectrum originates from radiation that has penetrated through the surface of the sample and has been transmitted through multiple particles. A relatively small fraction of the DR spectrum originates from radiation that has been reflected from the front surface of the samples and therefore has the shape of a Fresnel reflection spectrum. Because the shapes of bands in mid-IR Fresnel reflection spectra are asymmetrical, the fraction of Fresnel reflection that contributes to a DR spectrum should be reduced to be as small as possible. This may be achieved in a number of ways, the most important and commonly used of which is to dilute the sample by mixing it with 90%–99% of a nonabsorbing diluent such as finely powdered potassium bromide or potassium chloride. The sample dilution has the added benefit of reducing absorption band intensities to an appropriate level.

DR spectra largely result from photons that have been transmitted through tens to hundreds of particles and, therefore, have an appearance similar to that of transmission spectra. However, DR spectra do not obey Beer's Law. Instead, the DR spectrum measured at infinite depth, R_∞ (i.e., band intensities do not change if the thickness of sample is increased), is converted by the Kubelka–Munk function:

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$$f(R_\infty) = \frac{(1-R_\infty)^2}{2R_\infty} \qquad (4)$$

The function $f(R_\infty)$ is equal to the ratio of the absorption coefficient to the scattering coefficient of the sample. DR is usually calculated by taking the ratio of the single-beam spectrum of the diluted sample to the single-beam spectrum of the neat diluent. Ideally, the sample is ground to the point that the average particle diameter is <5 µm. The sample–diluent mixture is thick enough that any increase in its thickness does not lead to a change in the spectrum. Samples measured in this way are said to be measured at *infinite depth*, and the reflectance is given the symbol, R_∞. For mid-IR DR spectrometry, the infinite depth criterion is usually obeyed when the thickness is at least 100 µm, but to be conservative the depth of most sampling cups for mid-IR DR spectrometry is at least 1 mm.

A common way of preparing samples for DR spectrometry is to overfill the cup and to level the sample with a spatula or razor blade. However, this way of preparing the sample can lead to a difference between the scattering coefficient near the surface and in the bulk of the sample. Because the intensity of DR spectra depends on the scattering coefficient, a better sample preparation procedure is to slightly overfill the cup and to tap the base of the cup on a bench until the top surface is level with the rim of the cup.

Several types of accessories are used for the measurement of DR spectra. On-axis, or bright-field, DR accessories are similar to very efficient specular reflection accessories. They are the most efficient devices for the measurement of DR spectra but generally give the least rejection of Fresnel reflection. Off-axis, or dark-field, DR accessories are less efficient than on-axis devices but reject specular reflection more efficiently. Compound parabolic concentrators also have been adapted for DR measurements and appear to be intermediate in efficiency and Fresnel reflection between on-axis and off-axis accessories. Finally, integrating spheres have been used for DR spectrometry. These devices are the most accurate photometrically but are the least efficient.

MICROSPECTROSCOPY AND IMAGING

Transmission Microscopy

In any microscope, the area of a sample under study is defined by one or more remote masking apertures mounted at focused image planes conjugate to the sample focus plane. If the sample is mounted on a computer-controlled *x–y* stage, then successive neighboring or specified regions can be measured sequentially. These single-point spectra can be used to generate absorbance-intensity contour maps or false-color images that highlight differences or inhomogeneities across the area mapped. Such maps can also be generated by moving the sample manually, but this procedure can be very time consuming. The linear dimension of the smallest sample that can be studied by mid-IR FT-IR microscopy is approximately equal to the wavelength, i.e., approximately 10 µm. In practice, one can usually record a single-point spectrum of acceptable signal-to-noise ratio (SNR) and spectral resolution within a reasonable time-scale from a masked sample area of 10-µm diameter in about 1 min. For mapping over a relatively large sample area, where hundreds or even thousands of spectra are needed, a much more time-efficient process is to use an FT-IR microscope equipped with an array detector. Variable-temperature studies with thermomicroscopy observations and variable-temperature studies undertaken on an FT-IR microscope can be particularly useful for studying solid-state form and thermally induced transitions in situ.

As with macroscopic sampling, micro-samples presented for transmission FT-IR microscopy measurements should be flat. For a full mid-IR fingerprint spectrum, they should be of an appropriate thickness which, in the case of many APIs, may require a sample of approximately 10-µm thickness or less. Compression cells are particularly useful for thinning a sample. A thinned sample can be examined under compression or (less preferably) with the top window of the cell removed and the thinned sample examined while supported on the bottom window. A good practical means of providing an appropriate sample from which to record a single-beam background spectrum is to mount a small particle of potassium bromide alongside the sample and thin both by compression under the same conditions. The single-beam spectra of the sample and reference materials then can be measured under the same conditions simply by moving the compression cell using the *x–y* stage of the microscope.

Many continuous solids such as polymers used in packing or fibers found as contaminants can be prepared to an appropriate thickness and examined in a compression cell by FT-IR microscopy. Rolling with a tool specifically designed for this purpose can also sometimes decrease the thickness of soft samples. Laminated samples can be sectioned using a microtome and can be examined either free-standing or supported on an IR-transparent window. Analysts commonly use a microtome to get a cross-section from a multi-layer polymer laminate film so that its layer structure can be analyzed by FT-IR microscopy.

Reflection Microscopy

All major types of reflection spectroscopy discussed above can be implemented on microscopes. Most microscopes have optical configurations that enable them to be switched from transmission to reflection measurements if the operator switches a simple flip mirror. The angle of incidence for these measurements is usually between 30° and 45° so that both Fresnel reflection and transflection measurements can be carried out easily. In contrast, special microscope objectives are required for ATR or reflection–absorption spectrophotometry.

For ATR microspectroscopy, the sample under investigation is supported on the microscope stage, and the ATR objective is lowered until the IRE is in optical contact with the uppermost surface of the sample. Reproducible contact pressure can be achieved by the use of a pressure gauge. A disadvantage of using a germanium IRE is that, unlike zinc selenide, it does not transmit visible light, which precludes in situ visual inspection of the sample. ATR mapping using a computer-controlled mapping stage in a manner analogous to that of transmission mapping is also possible, but this is usually restricted to soft materials.

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Hyperspectral Imaging

The term *hyperspectral imaging* came into use to describe the process whereby a focal plane array (FPA) detector is used to record simultaneously an array of spectra from a stationary sample. Each pixel of the array records a spectrum of a different region of the sample. This approach is a much more time-efficient process than single-point mapping if many spectra are measured from a large sample area. Typical array sizes for mid-IR applications are 256 × 256, 128 × 128, and 64 × 64. The individual mercury cadmium telluride (MCT) detector elements (pixels) of most FPAs are 6.25 µm × 6.25 µm. The detectors that are incorporated in the FPA are photovoltaic (PV) detectors, unlike the single-element photoconductive (PC) MCT detectors that are used for many FT-IR measurements (see below). Whereas PC MCT detectors operate at least to 750 cm^{-1}, PV detectors that are installed in FPAs typically have a low wavenumber cut-off in the region of approximately 900 cm^{-1}. Advances in digital electronics and FPA design enable 64 × 64 MCT FPAs to be used with an FT-IR spectrophotometer operating in the more conventional continuous-scan mode. The three-dimensional array of data sets, two spatial and one spectral, recorded in such an imaging measurement has become known as a *hypercube* or data cube.

An alternative way of hyperspectral imaging that is now commercially available is a hybrid approach in which a linear array of small PC MCT detectors is used in combination with a computer-controlled mapping stage. Adjacent regions are rapidly repositioned under the array detector until the full spatial region of interest has been covered. The full field image is built up as a mosaic of the individual area images recorded. In one system, which incorporates a 16-detector linear array, the lower wavenumber cut-off is approximately 700 cm^{-1}.

INSTRUMENTATION

The majority of mid-IR spectra are measured with an FT-IR spectrophotometer. These instruments generally incorporate an incandescent silicon carbide (Globar®-type) source. The radiation emitted by the source is collimated and passed into a continuous scanning two-beam interferometer. The rate of change of optical path difference in the interferometer (usually known as the *optical velocity*) is typically on the order of 0.2–5.0 $cm \cdot s^{-1}$. The actual value depends on the detector and the analog-to-digital converter (ADC). The beam emerging from the interferometer is then focused at the center of the sample compartment of the spectrophotometer by an off-axis paraboloidal mirror. After being transmitted through, or reflected from, the sample, the beam is focused onto the detector. This signal is called an *interferogram*. The interferogram is a record of the variation of the AC component of the energy incident on the detector as a function of the optical path difference (retardation) of the interferometer. The Fourier transform of the interferogram is the single-beam spectrum.

The measurement usually involves passing a laser beam through the center of the interferometer, with the resulting sinusoidal interferogram measured by a detector of visible radiation at the same time the IR interferogram is measured by the IR detector. The usual laser used for this purpose is a helium–neon (HeNe) laser with a wavelength of 632.8 nm (15,802 cm^{-1}). This laser interferogram allows the exact position of the moving optical element in the interferometer to be determined. In FT-IR spectrophotometers equipped with an externally triggerable ADC, the IR signal is digitized at each wavelength of the laser interferogram, typically at the zero crossings. If the IR interferogram is sampled once per wavelength of a HeNe laser, the spectral range is restricted to 0–7901 cm^{-1}, i.e., one-half the wavenumber of the laser. In some contemporary FT-IR spectrophotometers, a sigma-delta ADC is used. These ADCs sample the interferogram at constant time intervals rather than at constant intervals of optical path difference, but they have a greater dynamic range than do externally triggered ADCs. In the latter case, the instrument's software calculates what the value of the interferogram would have been at the laser zero crossings.

In obtaining the spectrum of a sample, the single-beam spectrum of the sample and an appropriate reference are measured, and the ratio of these two single-beam spectra is calculated. If the spectrum is obtained in the transmission mode, the ratio is known as the transmittance spectrum, $T(\tilde{\nu})$. For many measurements, the negative logarithm of $T(\tilde{\nu})$ is calculated to give the absorbance, $A(\tilde{\nu})$. If the sample is interrogated in the reflection mode, the ratio is the reflectance spectrum, $R(\tilde{\nu})$. For ATR, transflection and reflection–absorption measurements, $R(\tilde{\nu})$, are usually converted to absorbance in the same way as for transmission spectroscopy. Conversion to $A(\tilde{\nu})$ is essential for quantitative measurements. For Fresnel reflection measurements, $R(\tilde{\nu})$ is often subjected to a Kramers–Kronig transformation to yield the absorption index spectra, $k(\tilde{\nu})$, and the refractive index spectra, $n(\tilde{\nu})$. For mid-IR diffuse reflection measurements, $R(\tilde{\nu})$ is usually converted by the Kubelka–Munk function (see *Equation 4*).

The standard detector used in FT-IR spectrophotometers is a room-temperature pyroelectric bolometer, most commonly deuterated triglycine sulfate (DTGS) or deuterated L-alanine-doped triglycine sulfate. These detectors respond to IR radiation of all wavelengths, and their low wavenumber cut-off is determined by the window behind which they are mounted. The window is typically selected to match the material on which the beamsplitter is deposited and is usually potassium bromide, so that the range is restricted to 400 cm^{-1}.

When the sensitivity of pyroelectric bolometers is inadequate, e.g., for measurements made through a microscope or with a gas chromatography interface, the more sensitive MCT detector is used. MCT detectors are generally operated in the photoconductive mode and are usually cooled to 77 K with liquid nitrogen (LN_2). Thermoelectrically cooled MCT detectors are available, but their low wavenumber cut-off is usually well above 1000 cm^{-1}, and they are not as sensitive as LN_2-cooled MCT detectors. Liquid nitrogen-cooled indium antimonide (InSb) detectors can also be used. These detectors are more sensitive than MCT but have a cut-off at 1800 cm^{-1}.

Two problems with MCT detectors should be noted. First, because they are more sensitive than DTGS detectors, they should be used only when the sample or sampling accessory attenuates the beam by at least a factor of 10. Otherwise, the ADC will be overloaded and the photometric accuracy of the spectrophotometer will be seriously affected. If the instrument is equipped only with an MCT detector (a rare circumstance), a neutral-density filter can be mounted in the spectrophotometer beam to reduce the energy of the beam at the detector to an appropriate level. Second, even when the ADC is not saturated, the response of MCT detectors is often nonlinear at high signal levels near the centerburst of the interferogram. The effect of this nonlinearity is to cause the baseline of the calculated single-beam spectrum to be displaced from zero. This effect can be seen readily when

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one plots the single-beam spectrum between 0 and 4000 cm^{-1}. If the average energy in the single-beam spectrum between, for instance, 400 and 300 cm^{-1} is above or below zero, the detector is responding in a nonlinear manner in the region of the centerburst, and the photometric accuracy of the measurement is degraded concomitantly. Some vendors supply software to correct for this effect, but in general the signal should be reduced by inserting a neutral-density filter, not an aperture stop, in the beam.

FACTORS THAT IMPACT MEASUREMENT PERFORMANCE

Spectral Resolution

The main factor that affects the resolution of an FT-IR spectrophotometer is the maximum optical path difference of the interferogram. The nominal resolution, $\Delta\tilde{\nu}$, is the reciprocal of the maximum optical path difference.

The divergence angle of the beam passing through the interferometer may also degrade the resolution. The effective collimation of the beam that passes through the interferometer is determined by the limiting aperture of the optical system. In instruments designed for high resolution ($\Delta\tilde{\nu} > 0.5$ cm^{-1}), an adjustable aperture that serves the same purpose as the entrance aperture of a monochromator is installed at a focus between the source and the interferometer. As the desired resolution is increased (i.e., $\Delta\tilde{\nu}$ is made numerically smaller), the diameter of this aperture, which is known as the *Jacquinot stop* or J-stop, is decreased, making the divergence angle of the beam in the interferometer smaller; however, this procedure may increase the noise in the spectrum. In lower resolution instruments (typically those with a maximum resolution of 1 or 2 cm^{-1}), the detector serves the purpose of the Jacquinot stop.

If the true full-width at half-height of the bands or lines in the spectrum is less than $\Delta\tilde{\nu}$, side lobes are seen on each narrow spectral feature. These side lobes can be eliminated by apodization, i.e., multiplying the interferogram by a function that is equal to 1 at the centerburst and decays monotonically with optical path difference. Besides reducing the amplitude of the side lobes, apodization also has the effect of degrading the resolution (broadening the bands). When an interferogram is not weighted, it is often (incorrectly) said to be apodized with a boxcar apodization function, although a better term would be a boxcar truncation function. The selection of apodization function should be made on the basis of the purpose of the experiment, but it is usually limited to a few choices.

The Norton–Beer weak, medium, and strong apodization functions give the optimum combination of side-lobe amplitude for a loss in resolution of 20%, 40%, and 60%, respectively. The Norton–Beer medium apodization function is generally appropriate for many measurements of condensed-phase samples. The Happ–Genzel apodization function is also a nearly optimal function that degrades the resolution by about 50% (i.e., it is midway between the Norton–Beer medium and strong functions). A commonly available function is the triangular apodization function. This function has the effect of causing significant deviations from Beer's Law and is not recommended.

Wavenumber Accuracy

The main factors that affect wavenumber accuracy are the alignment of the laser and IR beams in the interferometer and the divergence of the beam passing through the interferometer as observed with the IR detector. One might think that because the wavenumber of the laser beam is known very accurately, the wavenumber scale of a spectrum measured on an FT-IR spectrophotometer should be known to equal accuracy. However, the laser beam is highly collimated, whereas the beam from the IR source is not (a collimated beam can be obtained only from a point source). Because both the source and detector have a finite size, equal accuracy (of the wavenumber of the laser beam and the wavenumber scale of the spectrum measured) is never the case. As the diameter of the Jacquinot stop is decreased, a small wavenumber shift (always less than 0.25 $\Delta\tilde{\nu}$) will be observed. When the diameter of a sample is less than the diameter of the beam focus in the sample compartment or the sampling accessory, the effect is also to vignette the beam (i.e., to stop the beam down), and a small wavelength shift will be observed.

Photometric Accuracy

The main factor that affects the photometric accuracy of FT-IR spectrophotometers is the linearity of the detector response. As noted above, the response of pyroelectric bolometers usually varies linearly with the energy on the detector, but this is not the case for MCT detectors. With MCT detectors, the best way of detecting photometric error is measurement of the nonphysical energy in the single-beam spectrum below the detector cut-off.

Sensitivity

The sensitivity of the instrument can be determined by measuring two single-beam spectra under exactly the same conditions and calculating their ratio to produce what is commonly known as a 100% line. The noise level in different spectral regions can be estimated either as the peak-to-peak noise, i.e., the difference between the maximum and minimum values of the percent transmission in the selected spectral region(s), or the root-mean-square (RMS) noise, i.e., the standard deviation of the spectrum in that region. The RMS noise level is the preferred metric because this calculation involves all the data in the selected region rather than just the two most deviant points. An example of suitable measurement conditions to test the sensitivity of an FT-IR spectrophotometer equipped with a DTGS detector are 16 co-added scans, a resolution of 2 cm^{-1}, and Norton–Beer medium apodization. The most commonly used spectral region is 2200–2000 cm^{-1} because (a) this is where the performance of most mid-IR spectrophotometers is highest, and (b) no common atmospheric interferent such as water or carbon dioxide absorbs strongly in this region. However, other regions should be tested close to the ends of the spectrum, such as 650–450 cm^{-1} and 4000–3800 cm^{-1}. The SNR of the spectrophotometer operating with certain parameters in a given spectral region is estimated as 100/(RMS noise level in percent transmission).

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Beer's Law Linearity

For quantitative measurements, the spectrum is measured at a resolution that is at least twice as narrow as the narrowest band in the spectrum. The use of either the Norton–Beer medium or Happ–Genzel apodization function is recommended. For optimal photometric accuracy, the maximum absorbance of the analytical bands is no greater than 1.0 absorbance unit. Higher absorbance values can be tolerated with certain combinations of resolution and apodization functions. The effect of band width and peak absorbance on Beer's Law linearity for strong bands depends on the resolution and apodization function, and should be validated on a case-by-case basis.

Change to read:

▲⟨1856⟩ NEAR-INFRARED SPECTROSCOPY—THEORY AND PRACTICE

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1. THEORY

Near-infrared (NIR) spectroscopy is a technique with broad and varied applications in pharmaceutical analysis. The NIR spectral region lies between the ultraviolet (UV)-visible and infrared regions, and at one time was considered the forgotten region of the electromagnetic spectrum. It is a branch of vibrational spectroscopy that shares many of the principles that apply to other spectroscopic techniques. The NIR spectral region comprises two sub-ranges (see *Figure 1*) associated with detectors used in instrumentation. The short-wavelength (Herschel) region extends from approximately 780–1100 nm (12,821–9000 cm^{-1}), and the longer wavelengths, which fall between 1100 and 2500 nm (9000–4000 cm^{-1}), compose the traditional NIR spectral region. It is common to express the wavelength (λ) in nanometers (nm) and the frequency (ν) in reciprocal centimeters (wavenumber) as acquired by the instrument. Usually, Fourier-transform (FT) spectrometers report the *x*-axis in wavenumber (cm^{-1}), whereas a dispersive, monochromator-based instrument will show the *x*-axis in wavelength (nm).

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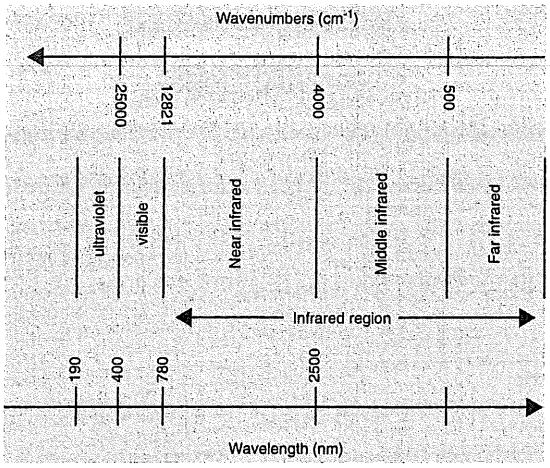

Figure 1. NIR region of the electromagnetic radiation spectrum.

NIR spectra are dominated by C-H, N-H, O-H, and S-H overtone resonances and combinations of fundamental mid-infrared vibrational modes from which they originate. Because molar absorptivities in the NIR range are low, radiation can penetrate several millimeters into materials, including solids where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an overtone) or the sum of two or more different modes (a combination band). Fiber-optic technology is readily implemented in the NIR range, which allows real-time, or near real-time, monitoring of processes in environments that might otherwise be inaccessible. As is the case with other spectroscopy measurements, interactions between NIR radiation and matter provide information that can be useful for both qualitative and quantitative assessment of the chemical composition of samples. In addition, qualitative and quantitative characterization of a sample's physical properties can be made because of the sample's influence on NIR spectra.

Measurements can be made directly on samples in situ in addition to applications during standard sampling and testing procedures. Applications of qualitative analysis include identification of raw material, in-process control testing, and finished-product release testing. NIR measurements can be performed off-line, but also at-line, in-line, and on-line for process analytical technology. These applications often involve comparing an NIR spectrum from a sample to a reference spectrum and assessing similarities against acceptance criteria developed and validated for a specific application. In contrast, applications of quantitative analysis involve the development of a predictive relationship between NIR spectral attributes and sample properties. Because of the highly covariant nature of the NIR signal, these applications typically use chemometrics models to quantitatively predict chemical and/or physical properties of the sample on the basis of its spectral attributes.

2. TRANSMISSION, REFLECTION, AND TRANSFLECTION MODES

The most common measurements performed in the NIR spectral range are transmission and reflection spectroscopy. Incident NIR radiation is absorbed or scattered by the sample and is measured as transmittance or reflectance, respectively.

2.1 Transmission Mode

Transmittance (T) is the intensity ratio of the transmitted radiation (I) to the incident radiation (I_0), which represents the decrease in intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and the detector. The arrangement is analogous to that in many conventional spectrophotometers, and the result can be presented directly in terms of transmittance and/or absorbance (A). NIR spectra are usually measured in absorbance (A).

$$T = \frac{I}{I_0} \text{ or } T = 10^{-A}$$

I = intensity of transmitted radiation
I_0 = intensity of incident radiation

The measurement of transmittance is dependent on a background transmittance spectrum for its calculation. Examples of background references include air, a polymeric disc, an empty cell, a solvent blank, or in special cases, a reference sample. The method generally applies to gases, liquids (diluted or undiluted), dispersions, solutions, and solids, including tablets and capsules. For transmittance measurements of solids, a suitable sample accessory is to be used. Tablets and capsules can be analyzed using suitably engineered holders, thereby ensuring reproducible measurements. Liquid samples are examined in a cell of suitable path length (typically 0.5–4 mm) that is transparent to NIR radiation, or alternatively by immersion of a fiber-optic probe of a suitable configuration. Gases are usually measured using dedicated long path-length, multiple reflection cells.

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2.2 Reflection Modes

2.2.1 REFLECTANCE

Reflectance (R) is the ratio of the intensity of light reflected from the sample, I, to that reflected from a background or reference reflective surface, I_R.

It is composed of both the specular (mirror) and the diffuse components (see *Figure 2*).

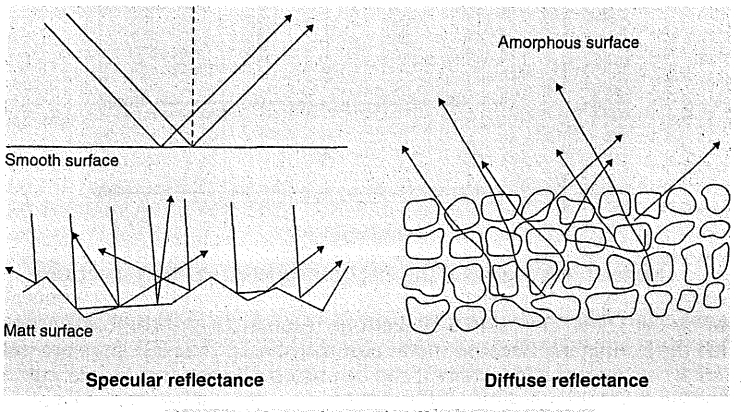

Figure 2. Specular and diffuse reflectances.

The determination of reflectance requires the measurement of a reference reflection spectrum to determine the attenuation of reflected light relative to the unattenuated incident beam. The reflectance spectrum is calculated as the ratio of the single-beam spectrum of the sample to that of the reference material.

$$R = \frac{I}{I_R}$$

I = intensity of light diffusively reflected from the sample
I_R = intensity of light reflected from the background or reference reflective surface

Most reflection measurements in the NIR spectral region are made of scattering samples such as powders and slurries. For such materials, NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an overtone) or the sum of two or more different modes (a combination band). Nonabsorbed radiation is scattered back from the sample to the detector. NIR reflectance spectra typically are accessed by calculating and plotting log ($1/R$) versus wavelength. Other abscissa data transformations can also be used. This logarithmic form is the pseudo-absorbance of the material and is commonly called absorbance.

2.2.2 DIFFUSE REFLECTION

The diffuse reflection mode gives a measure of reflectance (R) (penetrating the sample surface, interacting with the sample, and passing back through the sample's surface), which is the ratio of the intensity of light reflected from the sample (I) to that reflected from a background or reference reflective surface (I_r), where by using careful instrumental design, the specular component is often excluded. NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed by vibrational combinations and overtone resonances of the analyte species present in the sample. Nonabsorbed radiation is partially reflected back from the sample to the detector.

$$R = \frac{I}{I_r}$$

I = intensity of light diffusively reflected from the sample
I_r = intensity of light reflected from the background or reference reflective surface

This mode generally applies to solids. The sample is examined in a suitable device. For process monitoring, material can be analyzed through a polished glass (e.g., sapphire) window interface, or using an in-line probe. Care must be taken to ensure that the measuring conditions are as reproducible as possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references include ceramic tiles, thermoplastic resins, and gold. Other suitable materials may be used. In some process-analysis situations, it may be impossible to remove a probe for reference background data collection; in these cases, consider various options including internal referencing; measurement of a background reference using a second detector;

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and others. Only spectra measured against a background possessing the same optical properties can be compared directly with one another.

2.3 Transflection Mode

The term transflection (T^*) is used to describe any double-pass transmission technique. Transflection spectrometry is a hybrid of transmission and reflection wherein a reflector is placed behind the sample so that the optical path through the sample and back to the detector is doubled, compared with a transmission measurement of a sample of the same thickness. Nonabsorbed radiation is reflected back from the sample to the detector. The light may be reflected from a diffuse or specular (mirror) reflector placed behind the sample.

$$T^* = \frac{I}{I_{T^*}}$$

I = intensity of transflected radiation measured with the sample
I_{T^*} = intensity of transflected radiation of the reference material as background

This mode generally applies to liquids and clear plastic materials. This configuration can be adapted to share the same instrument geometry with reflectance and fiber-optic probe systems where the source and the detector are on the same side of the sample. The sample is examined through a cell with a mirror or a suitable diffusive reflector made of either metal or an inert substance (e.g., dried titanium dioxide) not absorbing in the NIR region. Liquids can also be measured using in-line transflectance probes to increase intensity levels for more sensitive applications.

3. FACTORS THAT AFFECT NIR SPECTRA

NIR spectroscopy is advantageous because, as a result of lower molar absorptivities in this region of the electromagnetic spectrum, accurate measurements can often be made quickly without destroying the sample, and with minimal or no sample preparation; this applies whether the samples are solid, semi-solid, liquid, or gas. The NIR spectrum contains information on overtone resonances and combination of fundamental vibrational modes of the sample that can yield both sample and process understanding. Samples may be analyzed directly through packaging or directly in media that is transparent to wavelengths in the NIR spectral region. In addition, by definition NIR spectra are produced using the corresponding NIR radiation, and therefore standard glass/quartz and fiber optics may be used.

The following discussion is not exhaustive, but it includes many of the major factors that affect NIR spectra.

3.1 Environmental Factors

The environmental temperature and humidity must be considered before carrying out measurements. Ideally, ambient conditions will be controlled to meet the operating specifications of the instrument manufacturer. However, tighter controls may not always be practical to the degree necessary for the application, and as such, another justifiable means of accounting for the influence of the environment (e.g., spectral preprocessing) should be utilized, with frequent verification. This is particularly relevant when considering the effect of temperature when using fiber-optic probes.

3.2 Sampling Factors

Depending on the measurement mode, sample preparation and presentation can vary. The following factors should be considered for all sampling techniques:

- Find the best suitable measurement mode for the intended application (transmission, diffuse reflection, or transflection)
- Find the best suitable accessory (e.g., transmission or immersion probes)
- Optimize path length in transmission and transflection modes
- Find a suitable spectroscopic background reference sample
- Show that the background reference sample is reliable over time and that the measurement of the background is reproducible and stable over time
- When measuring moving materials or samples (for process-related measurements), it is important to obtain a representative spectrum (e.g., by adjusting the measuring time or number of scans, by co-adding individual spectra, or by increasing the beam size)
- Ensure that there is not fouling of the sensor, for example, with build-up of material or contamination
- When measuring through packaging material, consider the variability of content and thickness
- Where multiple crystalline forms are present, care must be taken to ensure that the model calibration samples have a distribution of forms relevant to the intended application

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3.2.1 SAMPLE PRESENTATION AREA

The sample presentation area or probe end must be clean and free of residue prior to the measurement. Similarly, the in-line or on-line interface to the sample should not have significant product or contamination build-up, which would interfere with the desired measurement.

3.2.2 SAMPLE TEMPERATURE

Sample temperature influences spectra obtained from aqueous solutions and other hydrogen-bonded liquids, and a difference of a few degrees may result in significant spectral changes. Temperature may also affect spectra obtained from less polar liquids, as well as solids that contain solvents and/or water.

3.2.3 MOISTURE AND SOLVENT

Moisture and solvent that are present in the sample material and analytical system may change the spectrum of the sample. Both absorption by moisture and solvent, and their influence on hydrogen bonding of the active pharmaceutical ingredients and excipients, can change the NIR spectrum.

3.2.4 SAMPLE THICKNESS

Sample thickness is a known source of spectral variability and must be understood and/or controlled, particularly for tablet and capsule analysis. The sample thickness in transmission mode is typically controlled by using a fixed optical path length for the sample. In diffuse reflection mode, the sample thickness may be controlled by using samples that are "infinitely thick" relative to the detectable penetration depth of NIR light into a solid material. Here, the term "infinite thickness" implies that the reflection spectrum does not change if the thickness of the sample is increased. For the measurement of compressed powders, an infinite thickness is typically reached after 5 mm of sample depth (e.g., in a filled vial).

3.2.5 SAMPLE OPTICAL PROPERTIES

With solids, both surface and bulk scattering properties of model calibration standards and analytical samples must be taken into account. Surface morphology and refractive index properties affect the scattering properties of solid materials. For powdered materials, particle size and bulk density influence scattering properties and the NIR spectrum. The spectra of physically, chemically, or optically heterogeneous samples may require sample averaging, examination of multiple samples, or spinning the sample to obtain a representative spectrum of the sample. Certain factors, such as differing degree of compaction or particle size in powdered materials and surface finish, can cause significant spectral differences.

3.2.6 SOLID-STATE FORMS

The variations in solid-state forms (polymorphs, hydrates, solvates, and amorphous forms) influence vibrational spectra. Hence, different crystalline forms as well as the amorphous form of a solid may be distinguished from one another on the basis of their NIR spectra. Where multiple crystalline forms are present, care must be taken to ensure that the calibration samples have a distribution of forms relevant to the intended application.

3.2.7 AGE OF SAMPLES

Samples may exhibit changes in their chemical, physical, or optical properties over time. Depending on the storage conditions, solid samples may either absorb or desorb water/solvent, and portions of amorphous materials may crystallize. Materials used for NIR model calibration are representative of future samples and their matrix variables. Hence, care must be taken to ensure that samples for NIR analysis are representative.

4. PRETREATMENT OF NIR SPECTRAL DATA

NIR spectral data to be used in qualitative or quantitative applications often need preprocessing to attenuate environmental or sampling factors, enhance a certain signal, or for other purposes that may include transformation, normalization, or other mathematical treatment. Preprocessing of samples (rows of data) may include mean or median centering, scaling, and other procedures. NIR responses (columns of data) also can be transformed, centered, or scaled. Because of the highly covariant nature of the NIR signal, it is common to remove linear or polynomial trends or apply filtering or smoothing techniques such as derivatives, wavelets, or a Savitzky–Golay filter. Spectral pretreatment techniques may include the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended because of increased spectral noise. For more detail, see *Chemometrics* ⟨1039⟩.

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5. INSTRUMENTATION

5.1 Apparatus

All NIR measurements result from exposing material to incident NIR light radiation and measuring the attenuation of the emerging (transmitted, scattered, or reflected) light. Spectrophotometers for measurement in the NIR region consist of a suitable light source, such as a highly stable quartz–tungsten lamp, a monochromator or interferometer, and a detector. Common monochromators are acousto-optical tunable filters, gratings, or prisms. Traditionally, many NIR instruments have had a single-beam design, although some process instruments use internal referencing and can therefore be dual beam (for example, in diode array instruments). Silicon, lead sulfide, and indium gallium arsenide are examples of detector materials. Examples of sampling devices include conventional cuvette sample holders, fiber-optic probes, transmission dip cells, neutral borosilicate vials, and spinning or traversing sample holders. The choice is made on the basis of the intended application, with particular attention paid to the suitability of the sampling system for the type of sample to be analyzed. Suitable data processing and evaluation units (e.g., software and computer) are usually part of the system.

5.2 Imaging Techniques

NIR imaging is a combination of NIR spectroscopy with digital image processing. A NIR imaging system is basically composed of an illumination source, an imaging optic, a spectral encoder selecting the wavelengths, and a focal plane array. NIR imaging in particular has a huge potential for gaining rapid information about the chemical structure and related physical or biopharmaceutical properties of all types of pharmaceutical dosage forms, thus improving product quality and enhancing production speed.

Within a given material, the distribution of the various compounds can be characterized three-dimensionally by using imaging. Use of NIR imaging allows for the collection of detailed chemical information. NIR microscopy techniques have the following advantages:

- No preparation of the sample required
- Good spatial resolution
- Clear image quality
- Excellent chemical differentiation

It is possible to collect a signal from the entire sample (e.g., a tablet), disperse it into a spectrum using a spectrometer, and detect the spectrum using a multi-channel detector such as a charge couple detector or photo-diode array. In this case, instead of obtaining trivial cumulative information about the spot signal intensity, one can obtain a signal spectrum which can be transformed into detailed information about the chemical composition of the given spot on a sample. The three-dimensional array of data sets, two spatial and one spectral, recorded in such an imaging measurement has become known as a hypercube or data cube. These microscopy systems are capable of determining a spatial resolution down to approximately 1–5 µm.

NIR imaging systems are capable of characterizing the structure and distribution of the active components, and excipients within formulations, dosage forms, and delivery devices, to name a few. For example, chemical mapping for homogeneity testing of solid oral dosage forms, creams, and ointments may be useful in select applications.

5.3 Instrument Calibration Considerations

NIR instrument calibration involves three components: wavelength accuracy and uncertainty (*x*-axis); photometric linearity and response stability (*y*-axis); and photometric noise. *Table 1* provides an overview of performance verification criteria for a wide range of NIR systems.

5.3.1 PHOTOMETRIC NOISE

For any given method, the validation should include an estimation of the photometric noise of the system, as a change in this parameter may affect any future model calibration.

5.3.2 WAVELENGTH ACCURACY AND UNCERTAINTY (*X*-AXIS)

In the case of FT-NIR instruments, primary instrument wavelength-axis calibration is maintained, at least to a first approximation, with an internal helium–neon laser. NIR spectra collected by the conventional grating based spectra from sample and/or reference standard materials can be used to demonstrate an instrument's suitable wavelength-dispersion performance against target specifications. Suitable materials for demonstrating wavelength-accuracy performance include methylene chloride, talc *R*, spectral calibration lamps, polystyrene, mixtures of rare earth oxides, and absorption by water vapor. Certified traceable standards are available from the National Institute of Standards and Technology (NIST) for transmittance measurements (SRM 2035a) and reflectance (SRM 2036) and can be used for wavelength verification. Other suitable standards may also be used. Instrument manufacturers may use polystyrene films (thickness of 0.75–2 mm) as an internal standard for wavelength qualification and control. However, because of the weak asymmetric signals emanating from polystyrene at short wavelengths, this material may not be suitable as a reference standard across the whole NIR range. When choosing an appropriate reference standard, it is important to confirm that the operating parameters (e.g., resolution, band width, and others) are within the limits of the certified material.

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The effect of temperature on assignment of wavelength must be taken into account. Typically, standards are measured in environments near 25°C, and the temperature at which certified values were taken will be indicated. The effect of resolution can also have an impact on the measured values. Resolution for FT-NIR spectrometers is typically 8 cm^{-1}, but modern spectrometers are capable of higher resolutions (2 cm^{-1} or 1 cm^{-1}).

Standard certificates will indicate resolution and possibly even peak assessment algorithms. The choice of reference standard for a performance test should always match the resolution and environmental conditions expected for the execution of the test. Additional standards may be necessary, depending on the range of wavelengths or reference standards meeting these conditions.[1] Refer to *Table 1* and *Table 2* for additional information.

5.3.3 PHOTOMETRIC LINEARITY AND RESPONSE STABILITY (*Y*-AXIS)

Calibration of the photometric axis can be critical for successful quantification. Both FT-NIR and dispersive NIR spectrometers should undergo similar instrument calibration procedures. The tolerance of photometric precision acceptable for a given measurement should be assessed during the method development and validation stages.

NIR spectra from samples and/or reference standard materials with known relative transmittance or reflectance can be used to demonstrate a suitable relationship between NIR light attenuation (resulting from absorption) and instrument response. Verification of photometric linearity and verification of photometric noise are not required for instruments using methods to perform simple identifications that do not use the photometric absorbance as part of the model strategy (e.g., simple correlation with absorbing wavelengths). The USP Near IR System Suitability RS is recommended, but other certified traceable standards exist in the market and may be used. For reflectance measurements, commercially available reflectance standards with known reflectance properties are often used.

Spectra obtained from reflection standards are subject to variability because the experimental conditions under which they were factory calibrated differ from those under which they are subsequently put to use. Hence, the reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an "absolute" calibration for a given instrument. The reproducibility of the photometric scale will be established over the range of standards provided that 1) the standards do not change chemically or physically, 2) the same reference background is also used to obtain the standard values, and 3) the instrument measures each standard under identical conditions (including precise sample positioning). Subsequent measurements on the identical set of standards provide information on long-term stability. Refer to *Table 1* and *Table 2* for additional information.

Photometric linearity is demonstrated by using a set of transmission or reflection standards with known values for percentage transmittance or reflectance. For reflectance measurements, carbon-doped polymer standards are available. It is important to ensure that the absorbance of the materials used is relevant to the intended linear working range of the method. Nonlinear calibration models, which are acceptable, may be used, as long as the user can demonstrate adequate justification for this approach.

Table 1. Control of Bench/Mobile Instrument Performance

Measurement Mode	Reflection	Transflection	Transmission
	Measure talc via a suitable medium or by fiber-optic probe. Talc has suitable-for-calibration, characteristic peaks at 948, 1391, 2077, and 2312 nm. Alternatively, other suitable standards may also be used that ensure wavelength accuracy in the region of working methodology. For example, measure an internal polystyrene standard if built in, or measure an NIST standard or other traceable material (e.g., USP Near IR System Suitability RS) and assess 3 peaks across the wavelength range for calibration.	A suspension of 1.2 g of dry titanium dioxide in about 4 mL of methylene chloride is used directly through the cell or using a probe. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2500 nm (16 cm^{-1} at 4000 cm^{-1}). Methylene chloride has characteristic sharp bands at 1155, 1366, 1417, 1690, 1838, 1894, 2068, and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used, such as TS5 liquid mixed with titanium dioxide or some other reflective medium.	Methylene chloride may be used and has characteristic sharp bands at 1155, 1366, 1417, 1690, 1838, 1894, 2068, and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used, such as NIST SRM 2065, Polystyrene 65 µm, and TS5 liquid.
Verification of wavelength scale (except for filter apparatus)	For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapor line (for example, the line at 7306.74, 7299.45, or 7299.81 cm^{-1}).		
Verification of wavelength repeatability (except for filter apparatus)	The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified. Verify the wavelength repeatability using a suitable external or internal standard.		
Verification of photometric linearity and response stability[a]	Measure 4 photometric standards across the working method absorbance range. Analyze 4 reference standards, for example, in the range of 10%–90%, including 10%, 20%, 40%, and 80% with respective absorbance values of 1.0, 0.7, 0.4, and 0.1. Evaluate the observed absorbance values against the reference absorbance values (for example, perform a linear regression). Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the first verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values.		

[1] Burgess C, Hammond J. Wavelength standards for the near-infrared spectral region. *Spectroscopy*. 2007;22(4):40–48.

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Table 1. Control of Bench/Mobile Instrument Performance *(continued)*

Measurement Mode	Reflection	Transflection	Transmission
Verification of photometric noise	Determine the photometric noise using a suitable reflectance or transmittance standard (for example, white reflective ceramic tiles or reflective thermoplastic resins [i.e., polytetrafluoroethylene (PTFE)]). Follow the manufacturer's methodology and specifications. Scan the reflectance standard over a suitable wavelength range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise.		

[a] Burgess C, Hammond J. Wavelength standards for the near-infrared spectral region. *Spectroscopy.* 2007;22(4):40–48.

Table 2. Control of Process Instrument Performance

Measurement Mode	Reflection	Transflection	Transmission
Verification of wavelength scale (except for filter apparatus)	If it is not practically possible to measure a traceable standard material at the point of sample measurement, use internal material such as polystyrene, fiberglass, or solvent and/or water vapor. Alternatively, adopt a second external fiber/probe. For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapor line (for example, the line at 7306.74, 7299.45, or 7299.81 cm^{-1}) or a narrow line from a certified reference material.		
Verification of wavelength repeatability (except for filter apparatus)	The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified. Verify the wavelength repeatability using a suitable external or internal standard.		
Verification of photometric linearity and response stability[a]	Measure 4 photometric standards across the working method absorbance range.		
	If photometric reflectance standards cannot be measured at the point of sample measurement, use the manufacturer's internal photometric standards. Process instruments can use internal photometric standards for photometric linearity. Follow the manufacturer's verified tolerances in such cases.	Analyze 4 reference standards to cover the absorbance values over the working absorbance range of the modelled data. Evaluate the observed absorbance values against the reference absorbance values (for example, perform a linear regression). Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the first verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values.	
Verification of photometric noise	Determine the photometric noise using a suitable reflectance or transmittance standard [for example, white reflective ceramic tiles or reflective thermoplastic resins (i.e., PTFE)]. Follow the manufacturer's methodology and specifications.		
	As above, or if not practically possible, use the manufacturer's internal standard for noise testing and specifications.	Scan the transmittance high-flux standard over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise.	

[a] Burgess C, Hammond J. Wavelength standards for the near-infrared spectral region. *Spectroscopy.* 2007;22(4):40–48.

5.3.4 EXTERNAL INSTRUMENT CALIBRATION

Detailed functional validation employing external reference standards is recommended to demonstrate instrumental suitability of laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality-control procedures; rather, it provides independent documentation of the fitness of the instrument for the specific analysis or purpose. For instruments installed in a process location or in a reactor where positioning of an external standard routinely is not possible, including those instruments that employ an internal calibration approach, the relative performance of an internal versus an external calibration approach should be evaluated periodically. The purpose of this test is to check for changes or drifts in components (e.g., the process lens or fiber-optic probe) that might not be included in the internal calibration method; this could, for example, affect the photometric calibration of the optical system.

5.3.5 MULTI-INSTRUMENT CALIBRATION

There are many approaches for ensuring a transferable calibration across multiple instruments of the same type (same make and model) and different types. This is an area of growing interest because of the globalization of manufacturing and regulatory controls. Today, reference standards have been developed to cover this region, but many deficiencies still exist regarding wavelength uncertainty and photometric response curves. Therefore, verification of compendial procedures, method transfers, and spectrometer platform changes is compromised by the bias involved in differences between instruments. Understanding the risk associated with these variations should be a part of any quality-by-design initiative to use NIR spectroscopic procedures for product control.

The use of appropriate certified reference materials can be helpful in establishing instrumental variance and/or bias. As stated above, the value assignments included on the certification of such materials will contain significant contributions to the overall expanded uncertainty budget from the instrumental factors used in generating these values. For example, the optical geometry, polarization, and other aspects of a reflectance measurement will significantly affect the measured value, and therefore wherever possible these characteristics should be matched to the required measurement configuration. However, by definition, a reference material should have the essential characteristics of appropriate stability and homogeneity to at least allow comparative measurements to be made, albeit they may be biased by known or unknown factors.

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6. APPLICATIONS

NIR spectroscopy has a wide variety of applications for chemical, physical, and process analysis.

For chemical analysis, the applications include:

- Identification of active substances, excipients, dosage forms, manufacturing intermediates, chemical absorbance materials, and packaging materials
- Qualification of ingredients, intermediates, and drug products, including batch-to-batch spectral comparison and supplier change assessment
- Quantification of active substances and excipients; determination of chemical values such as hydroxyl value; determination of absolute water content; determination of degree of hydroxylation; and control of solvent content

For physical analysis, the applications include:

- Crystalline form and crystallinity, polymorphism, solvates, and particle size
- Analysis of intact pharmaceutical dosage forms: tablets, capsules, lyophilized products, and implants (e.g., polymeric and microspheres)
- Dissolution behavior, disintegration pattern, and hardness
- Examination of film properties

For process monitoring and process control analysis, the applications include:

- Monitoring of unit operations such as synthesis, crystallization, blending (e.g., powder), pelletization, tableting, capsule filling, drying, granulation, coating (e.g., film), and packaging for the purpose of process control

Measurements in the NIR spectral region are influenced by many chemical and physical factors. The reproducibility and relevance of the results depend on the control of these factors. Usually, measurements are only valid for a defined system.

6.1 Qualitative Analysis: Identification and Characterization

6.1.1 ESTABLISHMENT OF A SPECTRAL REFERENCE LIBRARY

To establish a spectral reference library, record the spectra of a suitable number of representative samples of the substance; the samples should have known, traceable identities. These representative samples should exhibit the variation that is typical for the substance to be analyzed (e.g., variation in solid-state form and particle size). The set of spectra obtained represents the information that can be used for chemical and/or physical identification of the sample to be analyzed.

The collection of spectra in the library may be represented in different ways, which are defined by the mathematical technique used for identification. These may be:

- All individual spectra representing the substance
- A mean spectrum of the measured batches for each chemical substance or physical form
- If necessary, a description of the variability within the substance spectra

The number of substances to be included in the library depends on the specific application. All spectra in the library will have the same spectral range and number of data points; technique of measurement; and data pretreatment. If subgroups (libraries) are created, the above criteria should be applied independently to each group. Origin spectral data for the preparation of the spectral library must be archived. Caution must be exercised when performing any mathematical transformation, as artifacts can be introduced or essential information can be lost. The suitability of the algorithm chosen should be demonstrated by successful method validation, and in all cases, the rationale for the use of transformation must be documented.

6.1.2 NIR REFERENCE SPECTRA

NIR references provide known, stable measurements to which other measurements can be compared; thus, they are used to minimize instrumental and environmental variations that would affect the measurement. Direct comparison of representative spectra of the substance to be examined and a reference substance for qualitative chemical or physical identification purposes may not require use of a reference spectral library, where specificity permits.

6.1.3 DATA EVALUATION

Direct comparison is made between the representative spectrum of the substance to be examined and the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification; alternatively, this can be achieved visually by overlaying spectral data if specificity is inherent. Different calibration techniques are available, such as principal component analysis, cluster analysis, etc.

6.1.4 VALIDATION OF THE MODEL

Chemical or physical identification methods using direct spectral comparison must be validated in accordance with identification method validation procedures. The validation parameters for qualitative methods are robustness and specificity.

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6.1.5 RELATIVE COMPARISON OF SPECTRA

A model calibration is not required when comparing a set of spectra for limit analysis purposes, such as determining the maximum or minimum absorbance at which an analyte absorbs. Also, in-process control of a drying operation may use a qualitative approach around a specific absorbing wavelength. Appropriate spectral ranges and pretreatments (if used) must be shown to be fit for purpose.

6.1.6 SPECIFICITY

The relative discriminatory power and selectivity of a limit test must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the measurement.

6.2 Trend Analysis

6.2.1 RELATIVE COMPARISON OF SPECTRA

NIR-based models can also be used to assess process trajectories. An example application is the use of NIR-based models to determine the endpoint of blending operation. Typically, a reference trajectory is defined based on a pool of batches that are deemed to present nominal variability; then each new batch trajectory gets compared with the reference trajectory and associated tolerances.

A model calibration is not necessarily required when comparing a set of spectra for trend analysis purposes, for example, using the moving-block approach to estimate statistical parameters such as mean, median, and standard deviation. For example, blend-uniformity monitoring using NIR spectroscopy has adopted such data analysis approaches. Appropriate spectral ranges and algorithms must be used for trend analyses.

6.2.2 SPECIFICITY

The relative discriminatory power and selectivity for trend analysis must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the trend analysis.

6.3 Quantitative Analysis

6.3.1 SPECTRAL REFERENCE LIBRARY FOR A CALIBRATION MODEL

Calibration is the process of constructing a mathematical model to relate the response from an analytical instrument to the properties of the samples. Any calibration model that can be defined clearly in a mathematical expression and gives suitable results can be used. Record the spectra of a suitable number of representative samples with known or future-established values of the attribute of interest (for example, content of water) throughout the range to be measured. The number of samples for calibration will depend on the complexity of the sample matrix and interferences (e.g., temperature, particle size, and others). It is encouraged to take a risk management approach to identify critical interferences that may need to be assessed. All samples must give quantitative results within a calibration interval as defined by the intended purpose of the method. Multiple linear regression, principal component regression (PCR), and partial least squares regression (PLS) are commonly used. For PLS or PCR calibrations, the regression coefficients and/or the loadings should be plotted, and the regions of large coefficients or loadings should be compared with the spectrum of the analyte. Predicted residual error sum of squares plots or similar plots are useful for facilitating the optimization of the number of PCR or PLS factors. For more information, see ISO 12099.

6.3.2 PRETREATMENT OF DATA

Wavelength selection, or exclusion of certain wavelength ranges, may enhance the efficiency of calibration models. Wavelength compression (wavelength averaging) techniques, multiplicative signal correction (MSC), standard normal variate transformation (SNV), and derivatives may be applied to the data.

6.3.3 MODEL VALIDATION PARAMETERS

Analytical performance characteristics to be considered for demonstrating the validation of NIR methods are similar to those required for any analytical procedure. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. Validation parameters for quantitative methods are accuracy, linearity over the operational range, precision (repeatability and intermediate precision), robustness, and specificity.

6.3.4 ONGOING MODEL EVALUATION

NIR models validated for use are subjected to ongoing performance evaluation and monitoring of validation parameters. If discrepancies are found, corrective action is necessary.

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7. PROCEDURE VALIDATION

Validation of NIR methods will follow the same protocols described in *Validation of Compendial Procedures* ⟨1225⟩ in terms of accuracy, precision, and other suitable parameters. It may be necessary to determine QL for methods of detection and quantification of an impurity or polymorphic form.

Detector linearity must be confirmed over the range of possible signal levels. Method precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids, and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample holder geometry, but will vary from instrument to instrument on the basis of excitation and optical configuration. In addition, many suitable chemometric algorithms for data pretreatment and calibration are available. Selection of an algorithm should be based on sound scientific judgment and suitability for the intended application.

7.1 Ongoing Method Evaluation

Validated NIR methods should be subject to ongoing performance evaluation, which may include monitoring accuracy, precision, and other suitable method parameters. If performance is unacceptable, corrective action is necessary. This involves conducting an investigation to identify the cause of change in method performance, and may indicate that the NIR method is not suitable for continued use. Improving the NIR method to meet measurement suitability criteria may require additional method development and documentation of validation experiments demonstrating that the improved method is suitable for the intended application. The extent of revalidation that is required depends on the cause of change in method performance and the nature of corrective action needed to establish suitable method performance. Appropriate change controls should be implemented to document ongoing method improvement activities.

Revalidation of a qualitative model may be necessary as a result of the following:

- Changes in instrument hardware
- Addition of a new material to the spectral reference library
- Changes in the physical properties of the material
- Changes in the source of material supply
- Identification of previously unknown critical attribute(s) of material(s)

Revalidation of a quantitative model may be necessary as a result of the following:

- Changes in the composition of the test sample or finished product
- Changes in the manufacturing process
- Changes in the sources or grades of raw materials
- Changes in the reference analytical method
- Major changes in instrument hardware

7.2 Method Transfer

Controls and measures for demonstrating the suitability of NIR method performance following method transfer are similar to those required for any analytical procedure. Any exceptions to general principles for conducting method transfer for NIR methods should be justified on a case-by-case basis. The transfer of an NIR method is often performed by using an NIR calibration model on a second instrument that is similar to the primary instrument used to develop and validate the method. When a calibration model is transferred to another instrument, procedures and criteria must be applied to demonstrate that the calibration model meets suitable measurement criteria on the second instrument. The selection of an appropriate calibration-model transfer procedure should be based on sound scientific judgment.

USP Reference Standards ⟨11⟩

USP Near IR System Suitability RS

GLOSSARY

[NOTE—This section refers to the technique of NIR in general. Please see *Analytical Instrument Qualification* ⟨1058⟩ and ⟨1039⟩ for additional information.]

Absorbance: Absorbance (A) is represented by the equation:

$$A = -\log T = \log (1/T)$$

where T is the transmittance of the sample. Absorbance is also frequently given as:

$$A = \log (1/R)$$

where R is the reflectance of the sample.

Background spectrum: This is used for generating a sample spectrum with minimal contributions from instrument response; it is also referred to as a "reference spectrum" or "background reference". The ratio of the sample spectrum to the background spectrum produces a transmittance or reflectance spectrum dominated by NIR spectral response associated with the sample. In reflection measurements, a highly reflective, diffuse standard reference material is used for the measurement of

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the background spectrum. For transmission measurement, the background spectrum may be measured with no sample present in the spectrometer or using a cell with the solvent blank or a cell filled with appropriate reference material.

Diffuse reflectance: Ratio of the spectrum of radiated light (penetrating the sample surface, interacting with the sample, and passing back through the sample's surface) reaching the detector to the background spectrum. This is the component of the overall reflectance that produces the absorption spectrum of the sample.

Fiber-optic probe: This consists of two components: optical fibers that may vary in length and number, and a terminus, which contains specially designed optics for examination of the sample matrix.

Installation qualification: The documented collection of activities necessary to establish that an instrument is delivered as designed and specified and is properly installed in the selected environment, which is suitable for the instrument's intended purpose.

Instrument bandwidth or resolution: A measure of the ability of a spectrometer to separate radiation of similar wavelengths.

Operational qualification: The process of demonstrating and documenting that an instrument performs according to specifications and that it can perform the intended task. This process is required following any significant change, such as instrument installation, relocation, or major repair.

Overall reflectance: The sum of diffuse and specular reflectance.

Performance qualification: The process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Performance qualification may employ the same or different standards for different performance characteristics.

Photometric linearity: Also referred to as "photometric verification", it is the process of verifying the response of the photometric scale of an instrument.

Pseudo-absorbance: A, is represented by the equation:

$$A = -\log R = \log (1/R)$$

where R is the diffuse reflectance of the sample.

Reference spectrum: See *Background spectrum.*

Reflectance: Is described by the equation:

$$R = \frac{I}{I_R}$$

in which I is the intensity of radiation reflected from the surface of the sample and I_R is the intensity of radiation reflected from a background reference material and its incorporated losses due to solvent absorption, refraction, and scattering.

Root-mean-square (RMS): Calculated using the equation:

$$\text{RMS} = \sqrt{\sum_{i=1}^{N} \frac{(\hat{A} - A_i)^2}{N}}$$

where $\hat{A}$ is the mean absorbance over the spectral segment; A_i is the absorbance for each data point; and N is the number of points per segment.

Specular (surface) reflectance: The reflectance from the front surface of the sample, where the angle of reflection matches the angle of incidence (mirror effect).

Standard error of calibration (SEC): A measure of the capability of a model to fit reference data. SEC is the standard deviation of the residuals obtained from comparing the known values for each of the calibration samples to the values that are calculated from the calibration. SEC should not be used as an assessment tool for the expected method accuracy (trueness and precision of prediction) of the predicted value of future samples. The method accuracy should generally be verified by calculating the *Standard error of prediction (SEP)* using an independent validation set of samples. An accepted method is to mark a part of the calibration set as the validation set. This set is not fully independent but can be used as an alternative for the determination of the accuracy.

Standard error of the laboratory (SEL): A calculation based on repeated readings of one or more samples to estimate the precision and/or accuracy of the reference laboratory method, depending on how the data were collected.

Standard error of prediction (SEP): A measure of model accuracy of an analytical method based on applying a given calibration model to the spectral data from a set of samples different from, but similar to, those used to calculate the calibration model. SEP is the standard deviation of the residuals obtained from comparing the values from the reference laboratory to those from the method under test for the specified samples. SEP provides a measure of the model accuracy to be expected when one measures future samples.

Surface reflectance: Also known as "specular reflection", it is that portion of the radiation not interacting with the sample but simply reflecting back from the sample surface layer (i.e., the sample–air interface).

Transflection: A transmittance measurement technique in which the radiation traverses the sample twice. The second time occurs after the radiation is reflected from a surface behind the sample.

Transmittance: Represented by the equation:

$$T = \frac{I}{I_0} \text{ or } T = 10^{-A}$$

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where I is the intensity of the radiation transmitted through the sample; I_0 is the intensity of the radiant energy incident on the sample ; and A is the absorbance. ▲ (USP 1-May-2020)

⟨1857⟩ ULTRAVIOLET-VISIBLE SPECTROSCOPY—THEORY AND PRACTICE

THEORY

Ultraviolet-visible (UV-Vis) spectroscopy is an electronic transition spectroscopic technique in which the interaction between incident radiation and electrons results in the promotion of one or more of the outer or the bonding electrons from a ground state into a higher-energy state. This quantum effect results in a specific absorption of radiation, the frequency and wavelength of which are governed by the equation:

$$E = h\nu = (hc/\lambda) \times 10^9$$

where:

E = energy
h = Planck's constant ($6.63 \times 10^{-34} J \cdot s$)
ν = frequency (Hz), related to the energy change ΔE, induced when electromagnetic radiation is absorbed ($\Delta E = h\nu$ per photon)
c = velocity of light (2.998×10^8 ms^{-1})
λ = wavelength (nm)

Even the simplest molecules have a large number of discrete energy levels and closely spaced levels adjacent to them caused by atomic vibration within the molecule. Overlap of these vibrational bands onto the electronic spectrum causes the measured spectra to appear as a broad, bell-shaped peak. As a general rule, most molecules absorb somewhere in the UV-Vis region. The greater the extent to which the p electrons are delocalized, the longer the wavelength of the first absorption band, i.e., the band of lowest energy and longest wavelength.

Derivative Spectroscopy

The advantages of derivative spectroscopy as an analytical tool have been known since the 1950s. Before the availability of the personal computer, generating derivative spectra electronically was complex and difficult, and for this reason the technique was rarely used. The introduction of microcomputers in the late 1970s simplified the generation of digital spectra and the associated mathematical manipulations required to produce first- and higher-order derivatives. This significantly increased the use of the derivative technique.

Derivative spectroscopy uses first or higher derivatives of absorbance (A) with respect to wavelength for qualitative analysis and for quantitation where:

$$A = f(\lambda) \text{ Zero order}$$

$$\delta A/\delta\lambda = f'(\lambda) \text{ First-order derivative}$$

$$\delta^2 A/\delta\lambda^2 = f''(\lambda) \text{ Second-order derivative}$$

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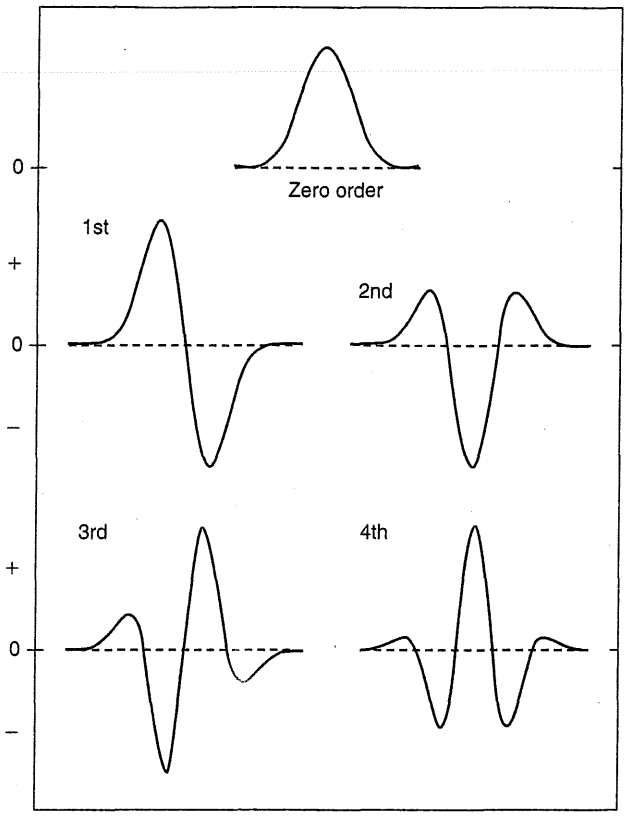

Figure 1. A Gaussian absorption band and its first to fourth derivatives.

A first-order derivative is the rate of change of absorbance with respect to wavelength (*Figure 1*). A first-order derivative starts and finishes at zero. It also passes through zero at the same wavelength as λ_{max} of the absorbance band. On either side of this point are positive and negative bands with a maximum and minimum at the same wavelengths as the inflection points in the absorbance band. This bipolar function is characteristic of all odd-order derivatives.

First-order derivatives are used principally for background absorption minimization or elimination for measurements on turbid, scattering solutions and suspensions and for analysis of trace components in complex absorbing matrices.

The most characteristic feature of a second-order derivative is a negative band with minimum at the same wavelength as the maximum on the zero-order band. A second-order derivative also shows two additional positive satellite bands on either side of the main band. A fourth-order derivative shows a positive band. A strong negative or positive band with a minimum or maximum at the same wavelength as λ_{max} of the absorbance band is characteristic of the even-order derivatives.

Higher-order (second and higher) derivatives can be used to:

- enhance resolution of overlapping peaks for the separation of superimposed spectra—particularly useful in multicomponent analysis
- assist quantitative determination of trace components
- aid characterization of individual pure compounds, particularly for archiving purposes and for complementing the information obtained from other techniques such as infrared, nuclear magnetic resonance, and mass spectroscopy
- assist in purity testing of products

Note that the minimum number of bands observed is equal to the derivative order plus one.

INSTRUMENTATION

All modern UV-Vis measurements involve detecting and measuring the intensity ratio of the radiation at a certain wavelength in the presence or absence of the absorbing sample. *Figure 2* is a schematic of a double-beam spectrophotometer. Dispersion of light to achieve the desired resolution can occur before or after introduction of the sample, but all commercial UV-Vis instruments share the following features to perform these functions:

- continuum source
- monochromator or polychromator
- sampling area
- detector

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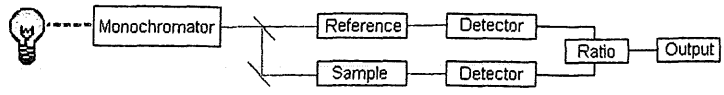

Figure 2. Typical double-beam spectrophotometer.

Care must be taken to ensure that the sample is not degraded by the incident light beam and that any heating effects are minimized. This is particularly important when diode-array instruments are used because the sample is irradiated at all wavelengths.

Continuum Source

Two major types of continuum source are currently in use: continuous and pulsed. Continuous sources include tungsten halogen for visible, deuterium arc for UV, and xenon arc for both. The source for pulsed radiation is the xenon flash lamp. Many UV-Vis instrument systems use a combination of deuterium and tungsten halogen to effectively cover the UV and visible regions, respectively. By necessity, source selection is achieved either by the use of a mirror or by physical movement of the lamps. This change usually is performed in the region of 320–350 nm, and thus qualification of the system must be performed using both source and mirror positions. Systems based on xenon lamps have the benefit of a single source and a higher energy output, but they are more expensive.

Monochromator

The wavelength scale can be encoded by either a scanning monochromator or a grating polychromator (see *Figure 3*), as is the case for spectrophotometers equipped with linear or two-dimensional array detectors. A discussion of the specific benefits and drawbacks of each of the dispersive designs is beyond the scope of this general chapter. Any properly qualified instrument should be suitable for qualitative measurements. Care must be taken when selecting an instrument for quantitative measurements because dispersion, response linearity, and stray light may not be uniform across the full spectral range.

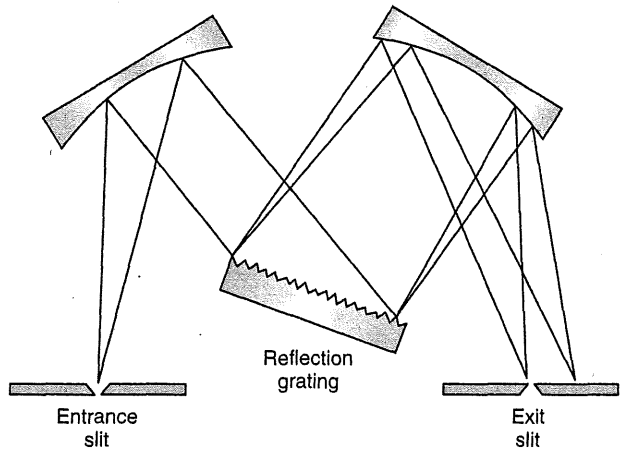

Figure 3. Grating monochromator.

Sampling Area

Numerous sampling arrangements are available in addition to the cell holders that are designed to accommodate various path-length configurations based on conventional rectangular cells. These include flow cells, fiber-optic-based immersion probes, micro-well plate configurations, and automated sample changers, among others. Considerations such as sampling volume, speed of measurement, and reproducibility of sample presentation should be evaluated to optimize the sampling device for specific applications.

Detector

Photoelectric detectors, which are the most common form of UV-Vis detectors, generate an electric current that is directly proportional to the intensity of the radiant energy incident upon them. They may take the form of photosensitive semiconductor devices, either discrete detectors, linear or two-dimensional arrays, or photomultipliers. Photosensitive semiconductor devices include solid-state photodiodes, charge-coupled device arrays (CCDs), and phototransistors. The most common semiconductor material is silicon, which is sensitive to wavelengths of 400–800 nm, but some silicon devices have extended sensitivity from as short a wavelength as 190 nm to as long a wavelength as 1100 nm. The dynamic response of these detectors typically is four orders of magnitude. Most array detectors are made of silicon and hence have a similar wavelength response. Other semiconductor materials can provide wavelength response to several micrometers.

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In contrast, photomultipliers are vacuum detectors that have a photocathode in which photon energy releases electrons that are directed by the field applied to electron-sensitive plates. By a cascade effect, these dynodes amplify the electrons released initially by the absorption of the incident radiation. Photomultipliers have typical wavelength responses of 160–900 nm, although some photocathode materials can provide response to higher wavelengths. The dynamic response of these detectors typically is six orders of magnitude or higher.

Alternative UV-Vis Detector Configurations

DIODE-ARRAY INSTRUMENTS

In a diode array, the optical configuration is reversed from that in a conventional spectrophotometer, and the light beam passes through the sample before being dispersed by the polychromator. This gives the benefit of fast, full spectral data with no moving parts that can wear out. The perception is therefore that diode arrays are more reliable than other detectors. This is true, but the reverse-optics design requires that both the sample and optics before the dispersing element (usually a grating) are subjected to the full-spectrum radiation also encountered by these components in a conventional spectrophotometer. In a conventional spectrophotometer, the sample and optics are outside the monochromator, and optical beam deflection and/or scattering simply reduces the intensity of the light that enters the monochromator. In an array-based spectrophotometer, deflection occurs within the monochromator, causing scatter within the confined environment and an associated increase in stray light that leads to a reduction in optimum photometric range.

HPLC DETECTORS

The design of a UV-Vis spectrophotometer is always the result of a number of considered compromises. For example, optical performance (absolute wavelength resolution) is governed by the focal length of the monochromator, which in turn dictates the physical size of the instrument. In the case of HPLC detectors the need to provide a high-stability, low signal-to-noise ratio output at high transmittance levels through a small-aperture flow cell requires that by design these systems may not have the dynamic range or wavelength accuracy of their conventional spectroscopic counterparts.

FIBER-OPTIC-BASED MODULAR SYSTEMS

In recent years there has been a rapid increase in the availability and use of systems built around the ability of fiber optics to channel and multiplex optical systems. Although these systems have the advantages of flexibility and ease of use and they allow measurements to be performed on micro-plates, customized systems, etc., analysts must consider the following disadvantages:

- Some analysts assume that these array-based fiber-optic systems are immune to room light interference at the sample interface, which may or may not be true. This assumption is easily tested by using a black photographic film-changing bag or a simple black cloth. Use of a masking technique should not change the measured value if the assumption is true.
- Custom-built systems do not have additional shuttering, stray light filtering, and other capabilities that are found in commercially designed spectrophotometers, and thus their performance characteristics, i.e., the optimum photometric range, may be significantly reduced.
- Light levels transmitted directly down fibers from high-intensity sources such as Xenon flash lamps may cause photodegradation. In simple self-built systems, the source may be coupled by fiber directly to the sample interface, and the only control is the source on/off switch.

CALIBRATION

UV-Vis instrument calibration involves two components: primary wavelength (*x*-axis) and intensity (*y*-axis), and often is performed when the instrument is initialized. Most dispersive UV-Vis instruments use atomic emission lines from either the source or a secondary lamp for primary wavelength (*x*-axis) calibration, in addition to the zero-order position in the monochromator. Depending on the vendor's configuration of the instrument, these calibration procedures may be available to the user. Calibration of the photometric scale (*y*-axis) is critical for successful quantitation and method transfer between instruments. Although the fundamental measurement relies on a simple ratio measurement, modern instruments may use several gain settings or amplification factors to ensure a linear detector response, particularly at low intensity levels and high power settings. These gain settings often are initially set by the manufacturer, but the settings may be recalibrated as the optics age or as other changes take place.

Detailed functional validation based on certified reference materials is recommended to demonstrate the suitability of laboratory instruments, even for instruments that possess an internal calibration capability. The use of external reference materials does not obviate the need for internal quality control procedures. Rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis.

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ANALYTICAL CONSIDERATIONS

Instrumental Factors

SPECTRAL BANDWIDTH

Because most spectrophotometric procedures require good spectral resolution, the spectral bandwidth is of great importance. Analysts should use the narrowest slit width that will provide an adequate signal-to-noise ratio because optimum resolution is achieved when the signal-to-noise ratio is maximized. If access to a variable-bandwidth instrument is available, then the optimum setting can be defined as the largest bandwidth at which no significant reduction in peak intensity is observed. In practical terms for molecules of pharmaceutical interest in solution, a spectral bandwidth of 2 nm is considered adequate.

STRAY LIGHT

Stray radiation, commonly referred to as stray light, can be defined as radiant energy at wavelengths other than those indicated by the monochromator setting and all radiant energy that reaches the detector without having passed through the sample or reference solutions (see *Figure 4*). It may be caused by any scattered radiation from imperfections in the dispersing medium, which commonly is a grating. The use of a holographic grating substantially reduces the levels of this source of stray radiation. Higher-quality ruled gratings yield a low level of scattered radiation. In higher-performance instruments, stray radiation can be reduced by the use of double monochromators or double-pass monochromators. Stray radiation, or apparent stray radiation, also may be caused by light leaks in the system, incorrect wavelength calibration, incorrect optical alignment, reduced source output, or reduced detector response.

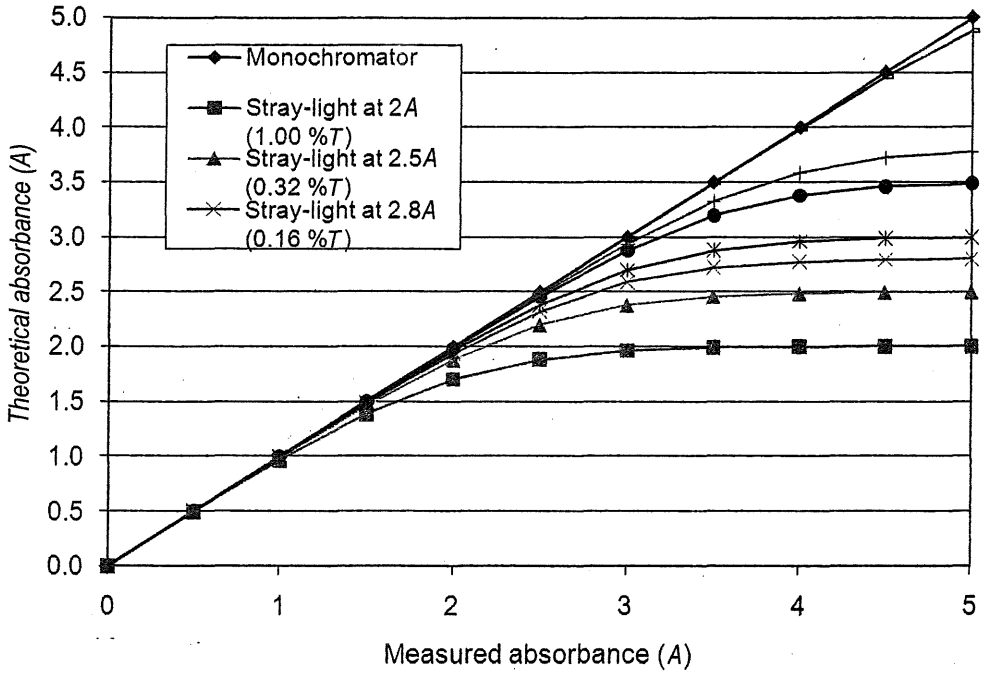

Figure 4. Effect of stray light on measured absorbance.

OPTIMUM WORKING PHOTOMETRIC RANGE

Determination of the optimum photometric range is fundamental to establishing the capability of a given instrument before and during method validation. The importance of this procedure can be shown by the fact that in 1945 Vandenbelt et al. (1) attempted to establish the optimum absorbance range on the then-new Beckman DU spectrophotometer. They suggested a simple approach in which the molar absorptivities of various compounds are measured at different concentrations. This approach is shown theoretically in *Figure 5*, where the center plateau is used to define the photometric range.

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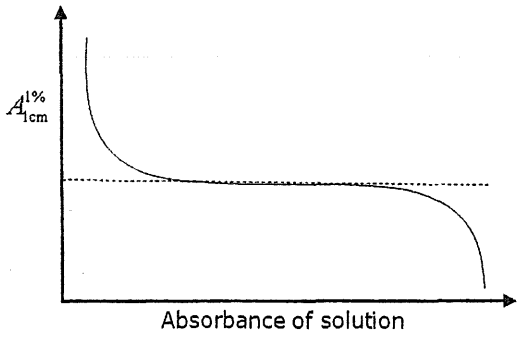

Figure 5. Theoretical photometric response curve.

In practice, data are more variable. *Figure 6* shows three typical curves.

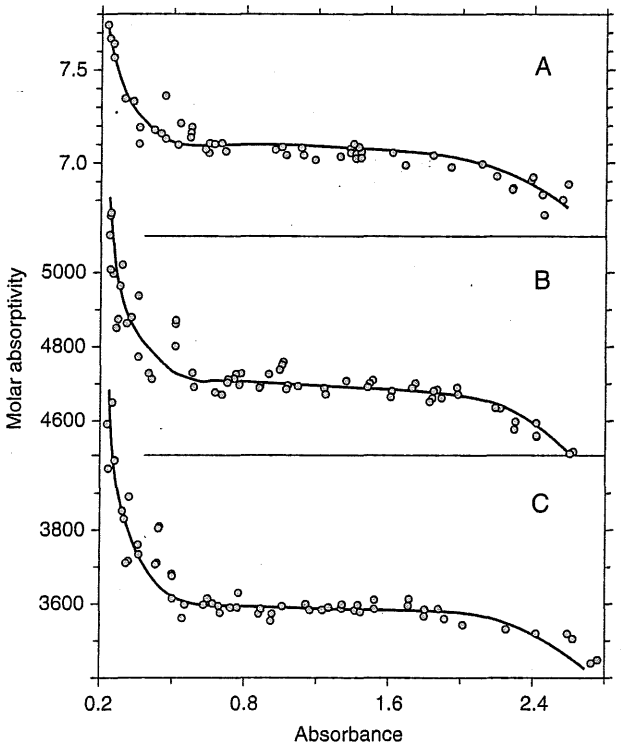

Figure 6. Molar absorptivity vs. absorbance for 3 compounds: (a) aqueous potassium nitrate measured at 301 nm, (b) potassium chromate in 50 mM potassium hydroxide at 373 nm, and (c) potassium chromate in 50 mM potassium hydroxide at 273 nm. Redrawn with permission from (2).

A more rigorous method for estimating an instrument's region of maximum precision is given by Youmans and Brown (3). The error in the measurement of the concentration of a solution of transmission T can be expressed as:

$$\Delta c = \frac{1}{\varepsilon b} \log \frac{T_{av}}{T_{min}}$$

where Δc is the change in concentration, ε is the molar absorptivity of the compound, b is the path length, and T_{av} and T_{min} are the mean and minimum values, respectively, from a series of n measurements on the same sample.

The statistical error is (see *Figure 7*):

$$\frac{\Delta c}{c} = \frac{1}{-\log T_{av}} \log \frac{T_{av}}{T_{min}}$$

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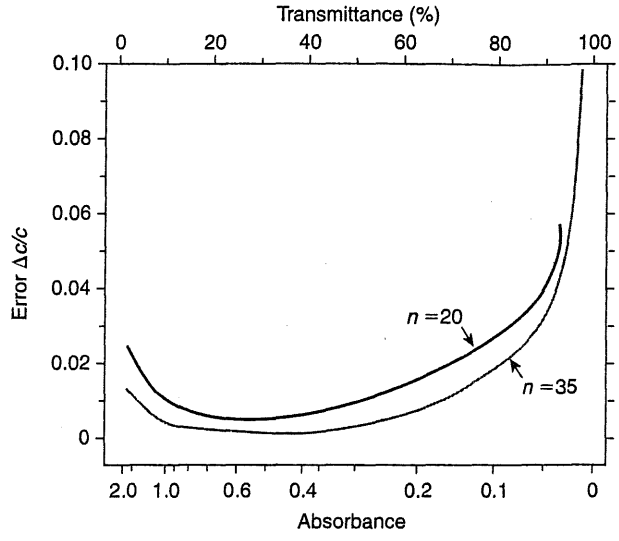

Figure 7. Youmans and Brown plot. Redrawn with permission from (2).

The noise levels, and hence the precision of measurement, depend primarily on detector noise type, as shown in *Figure 8*.

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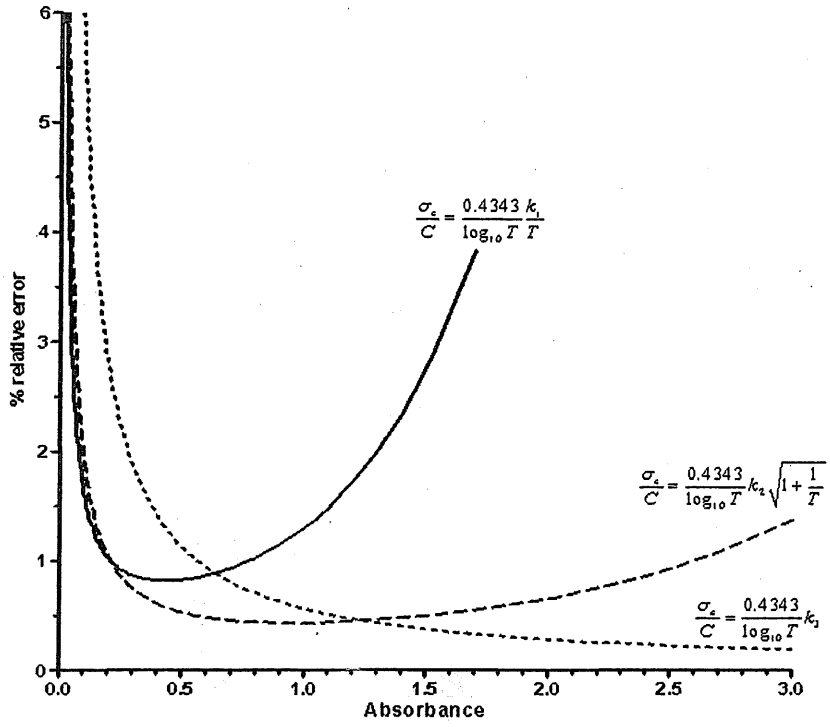

Standard deviation of a measurement σ_T	Source of variability	Relative error function
$\sigma_T = k_1$	Thermal detector, amplifier and dark current noise. [Independent of T]	$\frac{\sigma_c}{C} = \frac{0.4343}{\log_{10} T} \frac{k_1}{T}$
$\sigma_T = k_2 \sqrt{T^2 + T}$	Shot noise from the detector	$\frac{\sigma_c}{C} = \frac{0.4343}{\log_{10} T} k_2 \sqrt{1 + \frac{1}{T}}$
$\sigma_T = k_3 T$	Cell positioning, non-parallelism errors and incident beam intensity fluctuations	$\frac{\sigma_c}{C} = \frac{0.4343}{\log_{10} T} k_3$

Figure 8. Measurement precision as a function of noise type. Calculated and redrawn with permission from (4).

Both approaches clearly show that in addition to the expected increasing error at higher absorbance levels caused by stray light and other factors, there is a similar, if not larger possibility for increasing error at low absorbance levels caused by instrumental variances in the form of noise from the detector, source, etc. This variance often is overlooked in the desire for economy of scale, when analysts use smaller samples and shorter path lengths and therefore lower measured absorbance values.

Sample-Based Factors

The most important sample-based factors that negatively affect quantitative UV-Vis spectrophotometry are fluorescence and light scattering. If the sample matrix includes fluorescent compounds, the measured signal usually will contain a contribution from fluorescence. The wavelength range and intensity of the fluorescence depend on the chemical composition of the fluorescent material. Suspended particles scatter light by the Tyndall effect, causing a decrease in the measured intensity that increases as the wavelength decreases. Unless there is no alternative, absorbance should not be determined on turbid samples. Procedures for removing turbidity include filtering, centrifuging, or flocculating the sample and are performed before any additional procedures that generate a chromophore, provided that they do not affect the concentration of the analyte or the chromophore in the test solution. Any measurements performed on a turbid solution are highly instrument specific and can be used only for comparative purposes in the same system. A further complication can arise at higher analyte concentrations with respect to the coordination chemistry and system matrix. Ionic association, complex formation, and similar factors can cause deviation from the expected linear response.

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Sampling Factors

CELLS

Quantitative absorbance measurements usually are made on solutions of the substance in liquid-holding cells. The most common cell path length is 10 mm, although path lengths from 0.01 to 100 mm are commercially available (see *Figure 9*). For samples with low absorbance, improved sensitivity generally can be obtained by increasing the cell path length. For example, the theoretical absorbance of a solution in a 50-mm cell is greater by a factor of five compared to the same solution in a 10-mm cell. Errors in absorption readings arising from cells almost always are caused by dirty windows that can absorb a significant proportion of the incident light beam. Less frequent causes of error are an incorrect choice of cell material for the wavelength required, e.g., use of glass cells less than 320 nm, nonrepeatability of cell positioning, differences in cell window thickness, nonparallel optical windows, or impurities in cell window materials. A 10-mm cell manufactured with a ±0.05-mm tolerance will add a 0.005 *A* contribution to the overall uncertainty, when measuring absorbance values at the 1-*A* level. Ten-mm cells with a ±0.01-mm tolerance are commercially available. As a general guide, the cell chosen should have a transmittance value of >75% when filled with the appropriate solvent and measured at the wavelength of interest.

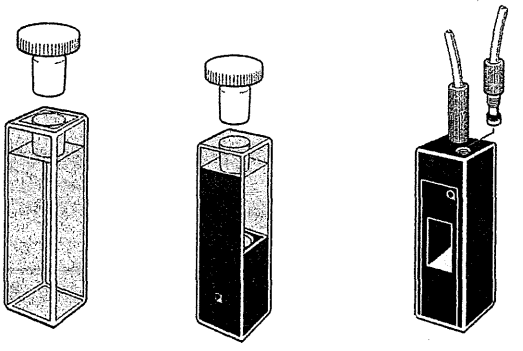

Figure 9. Commonly used cell types: 10-mm regular, low-volume, and flow cells (from left to right).

Analysts can mitigate the variability introduced by cells by adhering to the following set of best practices. Carefully clean cells before use, and store them in a manner that avoids contamination when they are not in use. Cells in frequent and repeated use, e.g., flow cells, can be stored wet in high-purity water or 1% (v/v) nitric acid. Do not use cracked or scratched cells. Wipe cells carefully with a soft, clean, lint-free cloth before they are placed in the spectrophotometer. Do not use lens-cleaning paper or any other form of paper cleaning tissue because these will either scratch or allow contamination of the optical face.

It is preferable to use a single cell for all measurements of both the reference and samples. When several sample cells must be tested against one reference cell, they all should be closely matched in their characteristics. Whenever possible, measure standards and samples at the same time and under the same conditions to minimize the potential for bias. The reference cell should contain the same solvent as the sample cell and should be checked against the sample cell at all wavelengths at which measurements are required. Analysts can apply corrections for differences between cells to absorbance readings obtained from the solutions under examination. The maximum allowable correction is ±0.01 absorbance unit. Best practices include using the same cell in the reference beam with the same face incident to the light beam, and flushing the sample cell three times with the sample solution before the final filling before measurement. The use of flow cells effectively addresses many of these cell-handling and cell-filling issues. The contents in both the sample cell and the reference cell must be free from gas bubbles and particles. If flow cells are used, analysts may be required to degas the solution(s) before measurement in order to avoid the formation of air bubbles in the cell.

A sequence of activities is used to monitor the quality of the measurement process and to ensure that the contents are representative of the sample under measurement. Drift of the spectrophotometric baseline can be detected by measuring the blank at the start and end of the sample sequence. Adequate flushing of the cell can be confirmed by alternating between measurement of the standard (high concentration) and the blank (zero concentration) at the start of the measurement sequence. The latter check is particularly important for automated systems that can generate reproducible but inaccurate results because of inadequate flushing.

CARE OF CELLS

Contaminated cells are the greatest single source of error in spectrophotometry. Cells should never be handled by the optical polished faces, and analysts should rinse off residual or spilled solution. If cells are properly cared for, rigorous cleaning methods rarely will be needed, especially if the cells are cleaned immediately after use. Although acid solutions will simply contaminate surfaces, alkaline solutions can etch all types of polished glass surfaces, and the degree of attack depends on the pH and contact time. When cleaning cells, analysts should ensure that the optical faces are not scratched or chipped. Commercial cleansing solutions are available and can be used, provided that they are diluted before use in accordance with the manufacturers' recommendations. After they are cleaned with such agents, the cells should be carefully and thoroughly rinsed with high-purity water. If this fails to clean the cells, then soaking them in cold, concentrated nitric or hydrochloric acid is acceptable. Flow cells are best cleaned in situ, provided that the solvent does not interact with the connecting tubing. If the cells are stored for an extended period, then they should be dried quickly after cleaning by blowing dry with a compressed gas stream in a clean, dust-free environment.

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ALIGNMENT AND FILLING OF CELLS

Analysts should align the cells so that the same optical face is always incident to the light beam. Most cells have type identification engraved on one face: this can be used to ensure consistent orientation. If there are no markings, a small mark can be made on one face outside the area of the light beam. The use of cells with no type markings is not recommended, but if they are used analysts should confirm their suitability for the intended application. If cells are provided with the instrument, individual cell holders should always be used in the same beam as identified, e.g., by use of appropriate markings.

CELL CORRECTIONS

If multiple cells are used, differences in the optical transparency of individual cells can introduce a systematic bias unless the latter is accounted for by a cell correction. Do not use corrections in excess of 0.01 absorbance unit. To determine if cell corrections are necessary, analysts can fill all cells with the appropriate solvent and measure the differences in absorbance at the required analytical wavelengths. These measurements are repeated after cleaning to ensure that differences in absorbance are related to the cells and are not the result of contamination. Analysts should fill and check the cells repeatedly until the results are consistent, and they should investigate any appreciable change in cell correction because it indicates contamination, damage to the cells, or incorrect adjustment of the instrument.

APPENDIX

Other Sources of Information

1. Vandenbelt, J.M., Forsyth, J. and Garrett, A. (1945) *Anal. Chem. (Industrial and Engineering Chemistry, Analytical Edition)*, 17(4), 1945, 235
2. Standards and Best Practice in Absorption Spectrometry, Ed. C. Burgess & T. Frost, Blackwell Science, (1999), ISBN 0-632-05313-5.
3. Youmans, H.L. and Brown, W.D. (1976) *Anal. Chem.*, 48, 1152.
4. Skoog, West & Holler (7th Ed) 1996, Table 24-4.

⟨1911⟩ RHEOMETRY

• **NEWTONIAN VISCOSITY**

If an external stress is exerted on a fluid, the latter will flow to a degree that is determined by internal friction forces derived from internal molecular interactions and by the magnitude of the external stress applied. The measure of resistance to flow is defined as the viscosity of the fluid. The coefficient of viscosity, η, historically termed the absolute viscosity, is defined by Newton's law of viscous flow:

$$\sigma = \eta \cdot \dot{\gamma} \qquad (1)$$

σ = shear stress, defined as the applied stress that causes successive parallel layers of a material body to move in their own planes relative to each other

$\dot{\gamma}$ = shear rate, defined as the rate of change of shear strain with time

It should be noted that although *stress* and *strain* are often used interchangeably, the terms are not used interchangeably in rheology: *stress* is synonymous with force per unit area or a system of forces per unit area, and *strain* refers to deformation (i.e., a change in shape or size). A fluid is said to exhibit Newtonian flow if the viscosity is a constant that is independent of the shear rate or applied shear stress. Because viscosity depends on temperature, the temperature of the substance being measured should be controlled to within ±0.1°.

In general, Newton's law is obeyed by liquids of low molecular weight and by solutions of low molecular weight solutes in low molecular weight solvents. However, liquids of high molecular weight, solutions that contain high molecular weight solutes and colloidal dispersions (e.g., suspensions and emulsions) typically do not obey Newton's law of viscous flow and are referred to as non-Newtonian fluids.

In the International System of Units (SI), the unit of viscosity is the pascal-second (Pa · s), which is equivalent to 1 kg · m^{-1} · s^{-1} or 10 Poise in the centimeter-gram-second (cgs) system. The viscosities of most Newtonian fluids are measured in millipascal-seconds (1 Pa · s = 1000 mPa · s; 1 mPa · s = 1 centipoise). Kinematic viscosity, ν, relates the viscous force to the inertial force by relating the Newtonian viscosity of a fluid to its density, ρ, at the same temperature:

$$\nu = \frac{\eta}{\rho} \qquad (2)$$

The units of kinematic viscosity in SI units are $m^2 \cdot s^{-1}$ or, more commonly, $mm^2 \cdot s^{-1}$. [NOTE—1 $mm^2 \cdot s^{-1}$ = 1 centistoke (cS or cSt). Stokes or centistokes are the units in the cgs system for kinematic viscosity.]

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Measurement of Newtonian Viscosity Using a Capillary Viscometer

The viscosity of a Newtonian fluid is measured by the use of glass capillary viscometers, examples of which are shown in *Figure 1* (simple U-tube capillary viscometer, also referred to as an Ostwald-type capillary viscometer) and *Figure 2* (constant or suspended-level viscometer, also referred to as an Ubbelohde-type capillary viscometer). [NOTE—When the term viscosity is used herein, it refers to absolute or Newtonian viscosity.] The constant- or suspended-level viscometer is a more robust design that is less influenced by temperature variations.

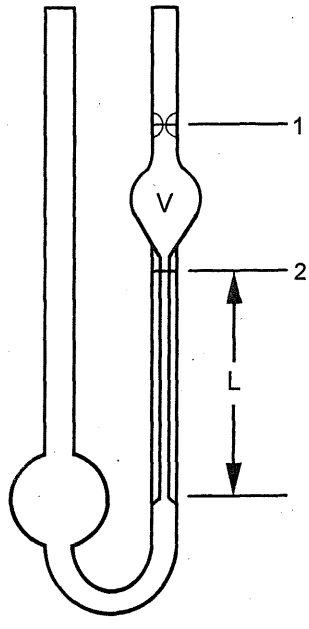

Figure 1. Simple U-tube (or Ostwald-type) capillary viscometer. [NOTE—Variables are defined in the text.]

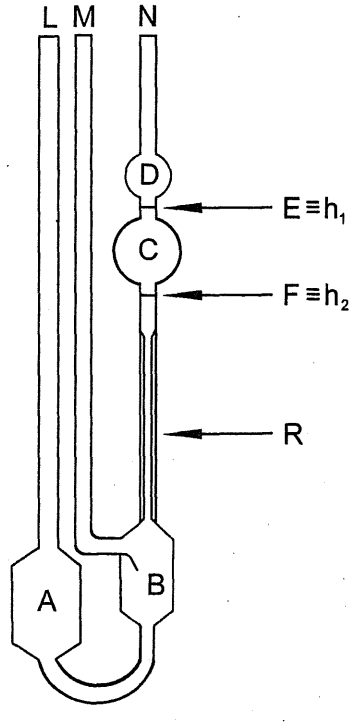

Figure 2. Suspended-level (or Ubbelohde-type) capillary viscometer. [NOTE—Variables are defined in Viscosity—Capillary Methods ⟨911⟩.]

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The following example is based on *Figure 1*, and the same principle is applicable to *Figure 2*. With the apparatus in *Figure 1*, the flow time, t, is measured for a given volume of fluid, V, as defined between lines 1 and 2 of *Figure 1*, to flow through a capillary tube having a length equal to L and a radius equal to r. As long as the density of the fluid, ρ, is known, the viscosity can be calculated using the Poiseuille equation:

$$\eta = \frac{\pi r^4 t \Delta P}{8LV} \qquad (3)$$

P = pressure

The applied pressure differential, ΔP, is defined as:

$$\Delta P = (h_1 - h_2) \times g \times \rho \quad (4)$$

h_1 = height of the fluid in the tube at line 1 measured from the bottom position
h_2 = height of the fluid in the tube at line 2 measured from the bottom position
g = gravitational acceleration (standard value = 9.80665 $m \cdot s^{-2}$)

Glass capillary viscometers generally are categorized as low shear stress instruments because ΔP is relatively small. The typical ranges for shear stress, σ, and shear rate, $\dot{\gamma}$ (at the wall), are 0.1–15 Pa and 100–20,000 s^{-1}, respectively.

The absolute dimensions of a capillary tube usually are too difficult to measure conveniently. As a result, an empirical calibration is employed for each viscometer tube. Collecting all the constants in *Equations 3* and *4* into a single viscometer constant, k, yields the approximate equation for kinematic viscosity:

$$\nu = k \times t \quad (5)$$

To determine k, analysts measure the flow time for a liquid of known viscosity at a given temperature. This result is used to calculate the viscometer constant for that particular temperature.

In work requiring a high degree of accuracy, analysts can use the following equation:

$$\nu = k \times t - (E/t^2) \quad (6)$$

E = kinetic energy factor, in $mm^2 \cdot s$

The viscometer constant, k, and kinetic factor, E, are determined from flow times measured for a set of stable, clean, Newtonian fluids of known kinematic viscosity (viscosity standard reference materials).

Once the viscometer constant(s) have been determined for a given temperature by the use of appropriate viscosity standards, the values can be used until the viscometer is subjected to thermal or mechanical stress that alters the dimensions of the capillary.

Generally, calibration, operation, and cleaning of viscometers should be performed according to instrument manufacturer's recommendations. Capillary viscometers must be scrupulously clean and should display no residual adsorbed or particulate organic or inorganic contamination. Surface contaminants may alter the dimensions of the capillary and the interfacial tension.

The procedures detailed in *Viscosity—Capillary Methods* ⟨911⟩ are intended for use with fluids that exhibit Newtonian behavior when subjected to shear stress. These procedures are not suitable for the characterization of non-Newtonian fluids because they may give erroneous and variable results if used with such fluids.

Capillary viscometers described in ⟨911⟩ typically measure rheological properties at only one shear rate during one pass of the sample through the capillary. Because of the nonlinearity of the shear rate–shear stress relationship for non-Newtonian fluids, this measurement is inadequate unless provisions are made for flow measurements at multiple rates of shear. Even more problematic for non-Newtonian fluids are the decrease in shear rate from the capillary wall to the center of the capillary and the variation in shear stress with time as the liquid level in the capillary viscometer decreases. Thus, capillary viscometers are inherently unsuitable for use in characterizing non-Newtonian fluids.

Because of the small ΔP attained with glass capillary viscometers, their use in measurements of high-viscosity fluids is impractical. Capillary-extrusion viscometers that use an external pressurized gas reservoir or other source of constant pressure are the only practical capillary instrumental alternative in these instances. In addition, capillary-extrusion viscometers may allow the operator to vary the shear rate or stress, thus enabling the characterization of non-Newtonian fluids. Chapter ⟨911⟩ does not address such devices.

Measurement of Newtonian Viscosity Using a Rotational Viscometer

The principle of the method is to measure the force (torque) acting on a rotor when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotational viscometers are used for measuring the viscosity of Newtonian fluids or the apparent viscosity of non-Newtonian fluids. Detailed methods and procedures for rotational instruments are provided in *Viscosity—Rotational Methods* ⟨912⟩. Measurement of Newtonian viscosity can be performed on rotational viscometers/rheometers following the methods and procedures proposed in *Viscosity—Rotational Methods* ⟨912⟩. The calculated viscosity of Newtonian fluids should be the same (within experimental error), regardless of the rate of shear (or rotational speed).

• **NON-NEWTONIAN RHEOLOGY**

Newton's law of viscous deformation or flow, described in the section on Newtonian viscosity, describes the relationship between an applied stress, σ, and the resultant flow with a rate of shear, or velocity gradient, of $\dot{\gamma}$. Disperse systems (i.e., suspensions or emulsions) or fluids that contain macromolecular components usually do not obey Newton's law. These exceptions to Newtonian behavior are the subject of this section. The categorization of such exceptions into different flow

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behaviors such as shear-thinning (pseudoplastic), shear-thickening (dilatant), Bingham, etc., is not rigid. Non-Newtonian fluids may exhibit different rheological behavior depending on shear rate, shear stress, and temperature. Time-dependent rheological behavior, such as rheopexy or thixotropy, is not considered in this chapter.

The rheological behavior of a non-Newtonian fluid is evident when shear stress is plotted as a function of shear rate. The shear stress–shear rate relationship for Newtonian compositions is linear and passes through the origin, whereas the shear stress–shear rate relationship for non-Newtonian fluids is nonlinear and may not pass through the origin, or it is linear but does not pass through the origin (Bingham body). Rheograms, or flow curves, of some typical non-Newtonian fluids are contrasted with those of Newtonian fluids in *Figure 3*.

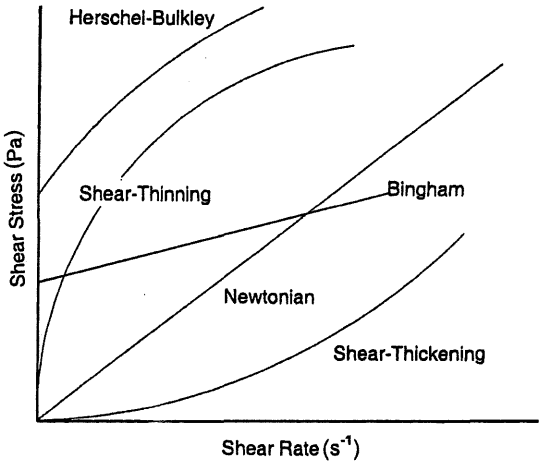

Figure 3. Rheograms of Newtonian and typical time-independent non-Newtonian fluids.

All the rheograms in *Figure 3* can be described by the Herschel–Bulkley equation:

$$\sigma = K \cdot \dot{\gamma}^{n} + \sigma_0 \qquad (7)$$

σ = shear stress, in Pa
K = consistency coefficient
$\dot{\gamma}$ = shear rate, in s^{-1}
n = the flow behavior index
σ_0 = yield stress, in Pa

For Newtonian fluids and Bingham plastics, K is designated as the viscosity (η) and plastic viscosity (η_{pl}), respectively. Because the viscosity of Newtonian fluids and the plastic viscosity of Bingham plastics are independent of the shear stress or shear rate, the flow behavior index, n, is equal to 1 for Newtonian fluids and Bingham plastics. For shear-thinning fluids, $0 < n < 1$, and for shear-thickening fluids, $1 < n < \infty$. Shear thinning can be observed in certain complex solutions such as aqueous hydrocolloid solutions. Dilatant behavior is generally exhibited by concentrated suspensions of nonaggregating monodisperse solid particles such as mixtures of corn starch and water. At very high viscosities or very low shear stresses, Bingham plastics exhibit a yield stress, σ_0, a shear stress below which no apparent deformation occurs (*Figure 3*). Other non-Newtonian fluids do not appear to exhibit a yield stress, σ_0, because flow occurs even at very low shear stresses. Thus, for non-Bingham bodies, in the absence of σ_0 the Herschel–Bulkley equation reduces to the power law, i.e., the Ostwald–de Waele equation:

$$\sigma = K \cdot \dot{\gamma}^{n} \qquad (8)$$

Many pharmaceutical macromolecular materials exhibit non-Newtonian behavior in solution. Although the viscosity of Newtonian fluids is a constant, i.e., $\eta = \sigma/\dot{\gamma}$ so that η is independent of shear rate or shear stress, there is no singular value of viscosity for non-Newtonian fluids because the viscosity of non-Newtonian fluids varies with shear rate or shear stress. Accordingly, applying the Newtonian concept of viscosity to non-Newtonian fluids is inappropriate. The viscosity value observed for a non-Newtonian fluid is an apparent viscosity, η_{app}, and is a function of the conditions of measurement. As the shear rate or shear stress changes, the apparent viscosity is likely to change as well. *Figure 4* depicts the dependence of η_{app} on the shear rate for an aqueous solution of a typical hydrocolloid.

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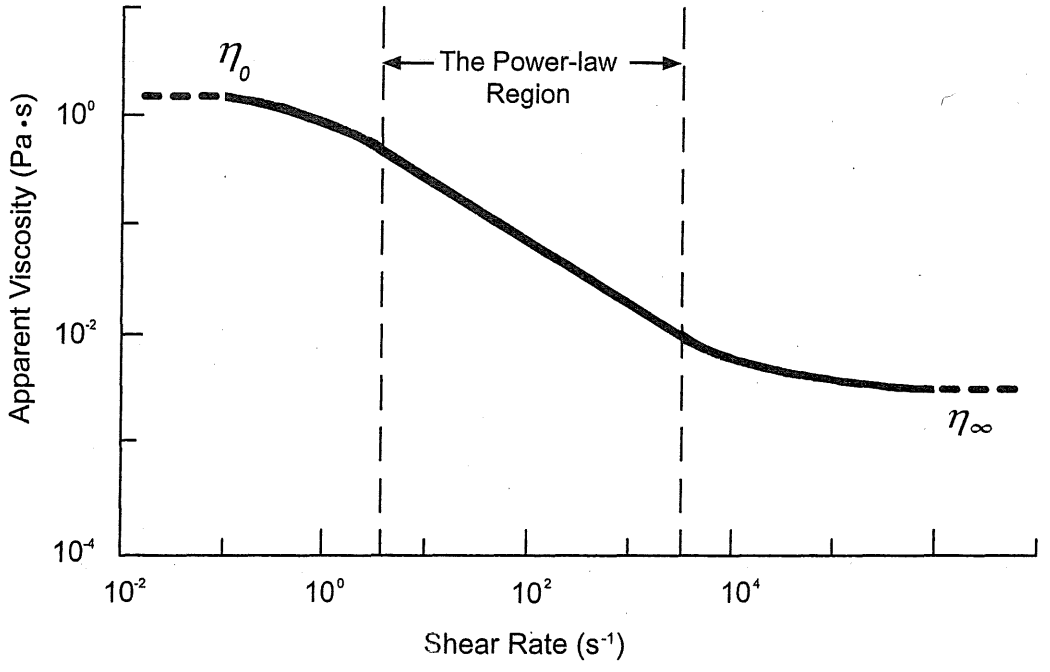

Figure 4. Log–log plot of the dependence of η_{app} on the shear rate for an aqueous solution of a typical hydrocolloid.

Figure 4 depicts apparent viscosities, η_{app}, ranging from high values at very low rates of shear to low values at very high rates of shear. The curve's asymptotes—corresponding to the zero-shear viscosity (η_0) and the infinite-shear viscosity (η_∞)—together with the linear log–log region at intermediate rates of shear, are characteristic of the shear-thinning behavior of these materials.

During the course of manufacturing, in-process materials that exhibit non-Newtonian behavior may be subjected to wide ranges of shear rates and shear stresses. Fluid dynamic conditions during drug product storage and administration and release of the active constituent(s) could broaden those ranges even further. For materials that exhibit non-Newtonian behavior in a solution or dispersion, the rheological profile (rheogram or flow curve), as described in *Figure 3*, gives a better functional characterization than a single viscosity measurement of the apparent viscosity at a single shear rate.

Determination of the rheological profiles of non-Newtonian solutions or dispersions requires the use of a rheometer or a viscometer that is capable of measuring rheological behavior over the range of shear rates and/or shear stresses encompassing the conditions that the excipient or product is likely to encounter. The exact mathematical characterization of deformation and flow of a test substance requires the precise delineation of sample dimensions, the forces exerted, and the resulting deformation or flow. Instruments that enable a thorough, geometrically accurate analysis of deformation and flow are termed *absolute* rheometers. Such absolute rheometers are preferred for measurements of non-Newtonian fluids so that results can be compared from instrument to instrument for corresponding ranges of shear stresses and shear rates. When flow or deformation is not well defined or is indeterminate, the resulting measurements are likely to correspond, at best, to approximations of the true rheological behavior of the sample. Rheometers that can provide useful information about material behavior, but do not enable the true rheological character of the material to be determined, are known as *relative* rheometers. Such instruments are subject to geometry-dependent limitations. Results obtained with relative rheometers generally are not comparable to those obtained with absolute rheometers or other differently configured relative rheometers. Relative rheometers often are called viscometers. However, the term *viscometer* should be reserved for a subset of rheometers that measure only steady shear flow and the corresponding Newtonian viscosity.

Temperature control during the course of measurement is critical to the rheological evaluation of non-Newtonian fluids. An Arrhenius-type equation has often been invoked to characterize Newtonian and non-Newtonian flow properties as a function of temperature:

$$\frac{\eta}{\eta_{ref}} = A \cdot e^{\left(\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right)} \qquad (9)$$

η = Newtonian or non-Newtonian viscosity at some absolute temperature T
η_{ref} = Newtonian or non-Newtonian viscosity at some reference temperature T_{ref}
A = constant
E_a = energy for viscous flow
R = universal gas constant

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Energies for viscous flow and the constant *A* are determined from experimental data. Energy dissipation by viscous flow is a function of measuring system geometry, temperature control, shear rate, and shear stress, but increased variability is observed in particular at high shear rates and for fluids that exhibit high apparent viscosity. Sample temperatures of non-Newtonian fluids should be controlled to ±0.1°, as is the case for Newtonian viscosity measurements.

Measurement of Viscosity Using a Rotational Rheometer

Rotational rheometers represent a more useful alternative to most capillary viscometers for measuring the rheological characteristics of non-Newtonian fluids as long as variations in shear rate, sample temperature, and edge or end effects can be minimized. The most common absolute rotational rheometer designs include concentric (coaxial) cylinder, cone-and-plate, and parallel-plate instruments. All of these are available in models that can provide absolute rheological data—that is, data that do not require substantial corrections for end or edge effects, viscous heating, or variations in shear rate or shear stress in the sample being evaluated. Rotating spindle rheometers—often described as viscometers—are relative rheometers that typically are used to assess apparent viscosity.

An additional factor with all rheometers/viscometers that must be taken into consideration, particularly with multiphase materials, is the potential formation of a thin, less-viscous layer of fluid at the wall of the instrument. When wall effects, or wall slip, occur, instrument measurements may be misleading because of the actual rheological behavior of the sample. Identification of wall effects is instrument specific. One useful method entails the comparison of results with a roughened vs. a smooth rotor. Instrument manufacturers generally provide recommendations for identification of and correction for wall slip.

Generally, calibration, operation, and cleaning of rheometers should be performed according to the instrument manufacturer's recommendations.

General chapter *Viscosity—Rotational Methods* ⟨912⟩ describes the procedures for the most common types of rheometers: spindle rheometers (viscometers) and concentric (coaxial) cylinder, cone-and-plate, and parallel-plate (parallel disk) instruments.

Calculation of shear rate, shear stress, and viscosity using a concentric (coaxial) cylinder rheometer:

For non-Newtonian liquids, it is essential to specify the shear stress, σ, or the shear rate, $\dot{\gamma}$, at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute rheometers), the shear rate $\dot{\gamma}$ in s^{-1}, and the shear stress σ, in Pa ($N \cdot m^{-2}$ or $kg \cdot m^{-1} \cdot s^{-2}$), are calculated using the following equations:

$$\dot{\gamma} = \left(\frac{R_o^2 + R_i^2}{R_o^2 - R_i^2} \right) \omega \qquad (10)$$

$$\sigma = \left(\frac{1}{4\pi h R_o^2} + \frac{1}{4\pi h R_i^2} \right) M \qquad (11)$$

R_o = radius of the outer cylinder (m)
R_i = radius of the inner cylinder (m)
ω = angular velocity (radian/s)
M = torque acting on the cylinder surface ($N \cdot m$)
h = height of immersion of the inner cylinder in the liquid medium (m)

Generally, the angular viscosity can be calculated using the equation:

$$\omega = \left(\frac{2\pi}{60} \right) n \qquad (12)$$

n = rotational speed, in revolutions per min (rpm)

For laminar flow, the viscosity η (or apparent viscosity η_{App}), in $Pa \cdot s$, is given by the following equation: [NOTE—1 $Pa \cdot s = 1000\ mPa \cdot s$.]

$$\eta \text{ or } \eta_{App} = \frac{1}{4\pi h} \left(\frac{1}{R_i^2} - \frac{1}{R_o^2} \right) \frac{M}{\omega} = k \frac{M}{\omega} \qquad (13)$$

k = constant of the apparatus (radians/m^3)

Calculation of shear rate, shear stress, and viscosity using a cone-and-plate rheometer:

The shear rate $\dot{\gamma}$ in s^{-1}, and the shear stress σ, in Pa, are calculated by the following equations:

$$\dot{\gamma} = \left(\frac{1}{\alpha} \right) \omega \qquad (14)$$

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$$\sigma = \left(\frac{1}{\frac{2}{3}\pi R^3} \right) M \qquad (15)$$

α = angle between the flat plate and the cone (radians)
R = radius of the cone (m)
ω = angular velocity (radian/s)
M = torque acting on the cylinder surface (N · m)

For laminar flow, the viscosity η (or apparent viscosity η_{App}), in Pa · s, is given by the following equation:

$$\eta \text{ or } \eta_{App} = \left(\frac{3\alpha}{2\pi R^3} \right) \left(\frac{M}{\omega} \right) = k \frac{M}{\omega} \qquad (16)$$

k = constant of the apparatus (radians/m³)

Measurement of Rheological Properties Using a Nonrotational Rheometer

Alternative nonrotational rheometer procedures for measuring rheological properties of non-Newtonian fluids include slit rheometers, small- and large-amplitude oscillatory shear-based rheometers, and capillary-breakup elongational rheometers. Equipment manufacturers generally provide detailed procedures, and these instruments and methods may be appropriate for use after users complete characterization and validation studies for both the equipment and the material under test. These types of rheometers are not described in *Viscosity—Rotational Methods* ⟨912⟩, as these are not rotational rheometers.

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Dietary Supplement Chapters

⟨2021⟩ MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS

INTRODUCTION

This chapter provides tests for the estimation of the number of viable aerobic microorganisms present in nutritional supplements of all kinds, from raw materials to the finished forms. Alternative methods may be substituted for the tests, provided that they have been properly validated as giving equivalent or better results. In preparing for and in applying the tests, observe aseptic precautions in handling the specimens. The term "growth" is used in a special sense herein, i.e., to designate the presence and presumed proliferation of viable microorganisms.

PREPARATORY TESTING

The validity of the results of the tests set forth in this chapter rests largely upon the adequacy of a demonstration that the test specimens to which they are applied do not, of themselves, inhibit the multiplication, under the test conditions, of microorganisms that may be present. Therefore, preparatory to conducting the tests on a regular basis and as circumstances require subsequently, inoculate diluted specimens of the material to be tested with separate viable cultures of the challenge microorganisms.

For the *Soybean–Casein Digest Agar Medium* used for *Total Aerobic Microbial Count*, inoculate duplicate plates with 25–250 cfu of *Staphylococcus aureus* (ATCC[1] No. 6538), *Escherichia coli* (ATCC No. 8739), and *Bacillus subtilis* (ATCC No. 6633) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For the *Sabouraud Dextrose Agar Medium* used for *Total Combined Molds and Yeasts Count*, inoculate duplicate plates with 25–250 cfu of *Candida albicans* (ATCC No. 10231) and *Aspergillus brasiliensis* (ATCC No. 16404) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For *Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)*, appropriate dilutions of *Escherichia coli* (ATCC No. 8739) and *Salmonella typhimurium* (ATCC No. 13311) are used. Failure of the organism(s) to grow in the relevant medium invalidates that portion of the examination and necessitates a modification of the procedure by (1) an increase in the volume of diluent, the quantity of test material remaining the same, or by (2) the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluents, or by (3) an appropriate combination of modifications to (1) and (2) so as to permit growth of the inoculum.

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in the preceding paragraph, using *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth under *Procedure* using the *Membrane Filtration Method* may be used.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above, and where the article is not suitable for the employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal or bacteriostatic activity of such magnitude that treatments are not able to remove the activity. This information serves to indicate that the article is not likely to allow proliferation or contamination with the given species of microorganism. Monitoring should be continued in order to determine the inhibitory range and bactericidal activity of the article.

[1] Available from ATCC, 10801 University Boulevard, Manassas, VA 20110-2209. Equivalent microorganisms, provided that they are from a national collection repository, can be used in lieu of ATCC strains. However, the viable microorganisms used in the test must not be more than five passages removed from the original ATCC or national collection culture.

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BUFFER SOLUTION AND MEDIA

Culture media may be prepared as follows, or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have similar ingredients and/or yield media comparable to those obtained from the formulas given herein.

In preparing media by the formulas set forth herein, dissolve the soluble solids in the water, using heat if necessary to effect complete solution, and add solutions of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at 25 ± 2°.

Where agar is called for in a formula, use agar that has a moisture content of NMT 15%. Where water is called for in a formula, use *Purified Water*.

pH 7.2 Phosphate Buffer

Prepare a stock solution by dissolving 34 g of monobasic potassium phosphate in about 500 mL of water contained in a 1000-mL volumetric flask. Adjust to a pH of 7.2 ± 0.1 by the addition of sodium hydroxide TS (about 175 mL), add water to volume, and mix. Dispense and sterilize. Store under refrigeration. For use, dilute the stock solution with water in the ratio of 1–800, dispense as desired, and sterilize.

Media

Prepare media for the tests as described below. Alternatively, dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of *Growth Promotion Testing*. Unless otherwise indicated elsewhere in this chapter, media are sterilized in autoclaves using a validated process. The exposure time within the autoclave at 121° will depend on the volume of media to be sterilized. Thus, for example, a 500-mL volume would need to be autoclaved using a temperature and time relationship that will ensure that the medium has attained at least an F_0 of 12–15 in the sterilization process. However, the appropriate time and temperature duration for sterilizing prepared media at any given volume should be confirmed by a thermal penetration study using a thermocouple or thermoprobe placed within the liquid medium.

FLUID CASEIN DIGEST–SOY LECITHIN–POLYSORBATE 20 MEDIUM

Pancreatic Digest of Casein	20 g
Soy Lecithin	5 g
Polysorbate 20	40 mL
Water	960 mL

Dissolve *Pancreatic Digest of Casein* and *Soy Lecithin* in 960 mL of water, heating in a water bath at 48°–50° for about 30 min to effect solution. Add 40 mL of *Polysorbate 20*. Mix, dispense as desired, and sterilize.

SOYBEAN–CASEIN DIGEST–AGAR MEDIUM

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.2.

FLUID SOYBEAN–CASEIN DIGEST MEDIUM

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose	2.5 g
Purified Water	1000 mL

Dissolve the solids in the water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that after sterilization it will have a pH of 7.3 ± 0.2. Filter, if necessary, and dispense into

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suitable containers. Sterilize at a temperature and time relationship that will ensure that the medium has attained at least an F_0 of 12–15 in the sterilization process, or by a validated filtration process.

SABOURAUD DEXTROSE–AGAR MEDIUM

Dextrose	40.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Agar	15.0 g
Water	1000 mL

Mix, and boil to effect solution.
pH after sterilization: 5.6 ± 0.2.

VIOLET-RED BILE AGAR WITH GLUCOSE AND LACTOSE

Yeast Extract	3.0 g
Pancreatic Digest of Gelatin	7.0 g
Bile Salts	1.5 g
Lactose	10.0 g
Sodium Chloride	5.0 g
D-Glucose Monohydrate	10.0 g
Agar	15.0 g
Neutral Red	30 mg
Crystal Violet	2 mg
Water	1000 mL

Adjust the pH so that it is 7.4 ± 0.2 after heating. Heat to boiling, but do not heat in an autoclave. Pour onto plates.

MOSSEL–ENTEROBACTERIACEAE ENRICHMENT BROTH

Pancreatic Digest of Gelatin	10.0 g
D-Glucose Monohydrate	5.0 g
Dehydrated Ox Bile	20.0 g
Monobasic Potassium Phosphate	2.0 g
Dibasic Potassium Phosphate	8.0 g
Brilliant Green	15 mg
Water	1000 mL

Suspend the solids in water, and heat to boiling for 1–2 min. Transfer 120-mL portions to 250-mL volumetric flasks or 9-mL portions to test tubes, all being capped with cotton plugs or loose-fitting caps. Heat on a steam bath for 30 min. Adjust the pH so that it is 7.2 ± 0.2 after heating.

GROWTH PROMOTION TESTING

Each lot of dehydrated medium bearing the manufacturer's identifying number or each lot of medium prepared from basic ingredients must be tested for its growth-promoting qualities. Cultures of *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 8739), *Bacillus subtilis* (ATCC No. 6633), *Candida albicans* (ATCC No. 10231), and *Aspergillus brasiliensis* (ATCC No. 16404) are used. A 10^{-3} dilution of a 24-hour broth culture of the microorganism to the first dilution (in *pH 7.2 Phosphate Buffer* or *Fluid Soybean–Casein Digest Medium*) may be used as the inocula. Serially streak plates of the media with the appropriate inocula to obtain isolated colonies to demonstrate the growth-promotion qualities of the *Soybean–Casein Digest Agar* and *Sabouraud Dextrose Agar* media. Inoculate the *Fluid Soybean–Casein Digest Medium* and *Mossel–Enterobacteriaceae Enrichment Broth* with 10–100 cfu of the appropriate challenge organisms to demonstrate their growth-promotion qualities.

SAMPLING

Provide 10-mL or 10-g specimens for the tests called for in the individual monograph.

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PROCEDURE

Prepare the specimen to be tested by a treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or part of it in a form suitable for the test procedure(s) to be carried out.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under *Total Aerobic Microbial Count*.

For a fluid specimen that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30% of alcohol, and for a solid that dissolves readily and practically completely in 90 mL of *pH 7.2 Phosphate Buffer* or the media specified, proceed as directed under *Total Aerobic Microbial Count*.

For water-immiscible products, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under *Total Aerobic Microbial Count*.

Total Aerobic Microbial Count

For specimens that are freely soluble, use the *Membrane Filtration Method* or *Plate Method*. For specimens that are sufficiently soluble or translucent to permit use of the *Plate Method*, use that method; otherwise, use the *Multiple-Tube Method*. With either method, first dissolve or suspend 10.0 g of the specimen if it is a solid, or 10 mL, accurately measured, if the specimen is a liquid, in *pH 7.2 Phosphate Buffer*, *Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to make 100 mL. For viscous specimens that cannot be pipeted at this initial 1:10 dilution, dilute the specimen until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipeted. Perform the test for absence of inhibitory (antimicrobial) properties as described under *Preparatory Testing* before the determination of *Total Aerobic Microbial Count*. Add the specimen to the medium NMT 1 h after preparing the appropriate dilutions for inoculation.

MEMBRANE FILTRATION METHOD

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield 30–300 colonies. Pipet 1 mL of the final dilution into 5–10 mL of *pH 7.2 Phosphate Buffer*, *Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium*. Wash each membrane with an appropriate amount of one of the above diluents. Transfer each membrane to a Petri dish containing *Soybean–Casein Digest–Agar Medium*, previously solidified at room temperature. Incubate the plates at a temperature 30°–35° for 48–72 h. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as "less than 10 microorganisms per g or per mL of specimen".

PLATE METHOD

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield 30–300 colonies. Pipet 1 mL of the final dilution onto each of two sterile Petri dishes. Promptly add to each dish 15–20 mL of *Soybean–Casein Digest–Agar Medium*, previously melted and cooled to about 45°. Cover the Petri dishes, mix the sample with agar by gently tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the Petri dishes and incubate for 48–72 h. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as "less than 10 microorganisms per g or per mL of specimen".

MULTIPLE-TUBE METHOD

Into each of 14 test tubes of similar size, place 9.0 mL of sterile *Fluid Soybean–Casein Digest Medium*. Arrange 12 of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set ("100") and into a fourth tube (A) pipet 1 mL of the solution or suspension of the specimen, and mix. Pipet 1 mL from tube A into the one remaining tube (B), not included in a set, and mix. These two tubes contain 100 mg or 100 µL and 10 mg or 10 µL of the specimen, respectively. Into each of the second set ("10") of three tubes pipet 1 mL from tube A, and into each tube of the third set ("1") pipet 1 mL from tube B. Discard the unused contents of tubes A and B. Close well, and incubate all of the tubes. Following incubation, examine the tubes for growth: the three control tubes remain clear, and the observations in the tubes containing the specimen, when interpreted by reference to *Table 1*, indicate the most probable number of microorganisms per g or per mL.

Table 1. Most Probable Count by Multiple-Tube Method

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			Most Probable Number of Microorganisms per g or per mL
Number of mg or µL of specimen per tube			
100	10	1	
3	3	3	More than 1100
3	3	2	1100
3	3	1	500

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Table 1. Most Probable Count by Multiple-Tube Method *(continued)*

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			Most Probable Number of Microorganisms per g or per mL
Number of mg or µL of specimen per tube			
100	10	1	
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23
2	2	0	21
2	1	1	20
2	1	0	15
2	0	1	14
2	0	0	9
1	2	0	11
1	1	0	7
1	0	0	4
0	1	0	3
0	0	0	<3

Total Combined Molds and Yeasts Count

PROCEDURE

Proceed as directed for *Membrane Filtration Method* or *Plate Method* under *Total Aerobic Microbial Count*, except to use the same amount of *Sabouraud Dextrose–Agar Medium* instead of *Soybean–Casein Digest–Agar Medium* and to incubate the plates for 5–7 days at 20°–25°.

RETEST

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10-g specimen, a retest on an additional 10-g specimen from the original sample and a 10-g specimen from the new sample of the nutritional supplement may be conducted. Proceed as directed under *Procedure*.

Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)

Dissolve or suspend the sample in a sufficient volume of *pH 7.2 Phosphate Buffer* or *Fluid Soybean–Casein Digest Medium* and dilute with *Fluid Soybean–Casein Digest Medium* to 100 mL. Pre-incubate for 2–5 h at 20°–25° in soybean–casein digest broth diluent; inoculate suitable quantities of *Mossel–Enterobacteriaceae Enrichment Broth* to contain 0.1, 0.01, or 0.001 g or mL of the product. Incubate at 30°–35° for 24–48 h. Subculture onto a plate of *Violet-Red Bile Agar with Glucose and Lactose*, and incubate at 30°–35° for 18–24 h. Growth of well developed, generally red or reddish, colonies of Gram-negative bacteria reveal the presence of enterobacteria. Determine the most probable number of microorganisms per g or per mL by reference to *Table 2*.

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Table 2. Most Probable Enterobacterial Count

Observed Presence of Enterobacteria			Most Probable Number of Enterobacteria per g or per mL
Number of g or mL of specimen per tube			
0.1	0.01	0.001	
+	+	+	More than 10^3
+	+	–	Fewer than 10^3 but more than 10^2
+	–	–	Fewer than 10^2 but more than 10^1
–	–	–	Fewer than 10^1

⟨2022⟩ MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS

INTRODUCTION

Good manufacturing practices require that objectionable organisms be absent from nonsterile nutritional and dietary products. A microorganism can be considered objectionable if it represents a potential health hazard to the user who is using the product as directed, or if it is capable of growing in the product. Objectionable microorganisms are defined as contaminants that, depending on the microbial species, number of organisms, dosage form, intended use, and patient population, would adversely affect product safety. Additionally, microorganisms may be deemed objectionable if they adversely affect product stability or if they may damage the integrity of the container closure system.

This chapter describes the testing of nutritional and dietary articles for specified microorganisms, which are specified in the individual monographs or whose absence is recommended by the guidance under *Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements* ⟨2023⟩. When objectionable microorganisms are not specified in the individual monograph, it is the manufacturers' responsibility to determine which microorganisms in their products are objectionable. It is not intended that all nonsterile nutritional and dietary articles be tested for the absence of all of the microorganisms mentioned in this chapter, nor is the testing of relevant microorganisms restricted to those presented in this chapter.

Alternative microbiological, physicochemical, and biotechnological methods, including automated methods, may be substituted for these tests, provided they have been validated as being equivalent in their suitability for determining compliance.

BUFFER AND MEDIA

General Considerations

See *Buffer Solution and Media* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩. The appropriateness of each medium for the intended purpose is to be assessed. Control sets of *Fluid Soybean–Casein Digest Medium* for *Preparatory Testing* are also used to assess the appropriateness of these media in the growth promotion of the specified microorganisms. For other media, streak agar plates to obtain isolated colonies of appropriate microorganisms, and inoculate the fluid media with the appropriate microorganisms at a final concentration of less than 100 cfu per mL. Observe the growth to establish the appropriateness of the media.

Buffer

BUFFER STOCK SOLUTION and pH 7.2 PHOSPHATE BUFFER

Proceed as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

Media

FLUID SOYBEAN–CASEIN DIGEST MEDIUM

Prepare as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

MANNITOL–SALT–AGAR MEDIUM

Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g

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Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Water	1000 mL

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.4 ± 0.2.

FLUID TETRATHIONATE MEDIUM

Pancreatic Digest of Casein	2.5 g
Peptic Digest of Animal Fat	2.5 g
Bile Salts	1.0 g
Calcium Carbonate	10.0 g
Sodium Thiosulfate	30.0 g
Water	1000 mL

Heat to boiling. Do not autoclave; use the same day. Immediately before use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat after adding the brilliant green solution.

BRILLIANT GREEN–AGAR MEDIUM

Yeast Extract	3.0 g
Peptic Digest of Animal Tissue	5.0 g
Pancreatic Digest of Casein	5.0 g
Lactose	10.0 g
Sodium Chloride	5.0 g
Sucrose	10.0 g
Phenol Red	80.0 g
Agar	20.0 g
Brilliant Green	12.5 mg
Water	1000 mL

Boil for 1 minute. Sterilize just prior to use, melt, pour into Petri dishes, and allow to cool.
pH after sterilization: 6.9 ± 0.2

XYLOSE–LYSINE–DESOXYCHOLATE–AGAR MEDIUM

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Desoxycholate (as Bile Salts)	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	800 mg
Water	1000 mL

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Heat, with swirling, just to the boiling point. Do not overheat or sterilize. Transfer at once to a water bath maintained at about 50°, and pour into Petri plates as soon as the *Medium* has cooled.
Final pH: 7.4 ± 0.2.

HEKTOEN ENTERIC AGAR MEDIUM

Protease Peptone	12.0 g
Yeast Extract	3.0 g
Lactose	12.0 g
Sucrose	2.0 g
Salicin	9.0 g
Bile Salts No. 3	9.0 g
Sodium Chloride	5.0 g
Sodium Thiosulfate	5.0 g
Ferric Ammonium Citrate	1.5 g
Acid Fuchsin	0.1 g
Bromothymol Blue	65 mg
Agar	14.0 g
Water	1000 mL

Mix, and allow to stand for 10 minutes. Heat gently, and allow to boil for a few seconds to dissolve the agar. Do not sterilize. Cool to 60°, and pour into Petri dishes.
Final pH: 7.5 ± 0.2.

TRIPLE SUGAR–IRON–AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Pancreatic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous Ammonium Sulfate	200 mg
Sodium Chloride	5.0 g
Sodium Thiosulfate	200 mg
Agar	13.0 g
Phenol Red	25 mg
Water	1000 mL

pH after sterilization: 7.3 ± 0.2.

MACCONKEY AGAR MEDIUM

Pancreatic Digest of Gelatin	17.0 g
Pancreatic Digest of Casein	1.5 g
Peptic Digest of Animal Tissue	1.5 g
Lactose	10.0 g
Bile Salts Mixture	1.5 g
Sodium Salts Mixture	5.0 g
Agar	13.5 g
Neutral Red	30 mg
Crystal Violet	1.0 mg
Water	1000 mL

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Boil for 1 minute to effect solution.
pH after sterilization: 7.1 ± 0.2.

LEVINE EOSIN–METHYLENE BLUE–AGAR MEDIUM

Pancreatic Digest of Gelatin	10.0 g
Dibasic Potassium Phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene Blue	65 mg
Water	1000 mL

Dissolve pancreatic digest of gelatin, dibasic potassium phosphate, and agar in water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, and add the remaining ingredients, as solutions, in the following amounts: for each 100 mL of the liquefied agar solution, add 5 mL of lactose solution (1 in 5), 2 mL of the eosin Y solution (1 in 50), and 2 mL of methylene blue solution (1 in 300). Mix. The finished *Medium* may not be clear.
pH after sterilization: 7.1 ± 0.2.

BAIRD–PARKER AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Beef Extract	5.0 g
Yeast Extract	1.0 g
Lithium Chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium Pyruvate	10.0 g
Water	950 mL

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg yolk emulsion prepared as follows. Disinfect the surface of whole-shell eggs, aseptically crack the eggs, transfer intact yolks to a sterile graduated cylinder, add sterile saline TS to obtain a 3 to 7 ratio of egg yolk to saline, add to a sterile blender cup, and mix at high speed for 5 seconds. Mix all ingredients well but gently, and pour into plates.
pH after sterilization: 6.8 ± 0.2.

VOGEL–JOHNSON AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Yeast Extract	5.0 g
Mannitol	10.0 g
Dibasic Potassium Phosphate	5.0 g
Lithium Chloride	5.0 g
Glycine	10.0 g
Agar	16.0 g
Phenol Red	25.0 mg
Water	1000 mL

Boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 mL of sterile potassium tellurite solution (1 in 100).
pH after sterilization: 7.2 ± 0.2.

FLUID SELENITE–CYSTINE MEDIUM

Pancreatic Digest of Casein	5.0 g
Lactose	4.0 g

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Sodium Phosphate	10.0 g
Sodium Acid Selenite	4.0 g
L-Cystine	10.0 g
Water	1000 mL

Mix, and heat to effect solution. Then heat in flowing stream for 15 minutes. Do not sterilize.
Final pH: 7.0 ± 0.2.

REINFORCED MEDIUM FOR CLOSTRIDIA

Beef Extract	10.0 g
Peptone	10.0 g
Yeast Extract	3.0 g
Soluble Starch	1.0 g
Glucose Monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium Chloride	5.0 g
Sodium Acetate	3.0 g
Agar	0.5 g
Water	1000 mL

Dissolve agar in water by heating to boiling, while stirring continuously. Adjust the pH if necessary, and sterilize.
pH after sterilization: 6.8 ± 0.2.

COLUMBIA AGAR

Pancreatic Digest of Casein	10.0 g
Meat Peptic Digest	5.0 g
Heart Pancreatic Digest	3.0 g
Yeast Extract	5.0 g
Cornstarch	1.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Dissolve agar in water by heating to boiling and with continuous stirring. If necessary, adjust the pH. Sterilize, and allow to cool to 45° to 50°. Add, when necessary, gentamicin sulfate, equivalent to about 20 mg of gentamicin base, and pour into Petri dishes.

Pre-reduction of the medium is recommended.
pH after sterilization: 7.3 ± 0.2.

RAPPAPORT VASSILIADIS SALMONELLA ENRICHMENT BROTH

Soya Peptone	4.5 g
Magnesium Chloride Hexahydrate	29.0 g
Sodium Chloride	8.0 g
Dipotassium Phosphate	0.4 g
Potassium Dihydrogen Phosphate	0.6 g
Malachite Green	0.036 g
Purified Water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°.
The pH is 5.2 ± 0.2 at 25° after heating and autoclaving.

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PREPARATORY TESTING

Proceed as directed for *Preparatory Testing* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

For enrichment broth, selective media, and differential media use an inoculating loop to transfer the inoculum of each test organism to the plated or liquid media being tested. If a plated medium is being tested, streak the surface of plate with the loop in four directions to obtain a pattern of isolated colonies. Incubate the media, and examine the plated or liquid media for the characteristic growth of the inocula (See *Tables 1*, *2*, *3*, and *4*).

SAMPLING

Proceed as directed for *Sampling* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

TEST PROCEDURES

Test Preparation—Prepare as directed for *Sampling*. Transfer to a suitable container with 100 mL of *Fluid Soybean–Casein Digest Medium (FSCD)*. Mix by shaking gently. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate.]

Test for Absence of *Staphylococcus aureus*

Incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from *FSCD* onto the surface of one or more of the following media: *Vogel–Johnson Agar Medium (VJ Agar)*, *Mannitol–Salt–Agar Medium (MS-Agar)*, and *Baird-Parker Agar Medium (BP Agar)*. Cover the Petri plates, invert them, and incubate at 30° to 35° for 24 to 48 hours.

Examine the plates of *VJ Agar*, *MS-Agar*, and/or *BP Agar*, and interpret the results with reference to *Table 1*: if no plate contains colonies having the characteristics described, the test specimen meets the requirement for the absence of *Staphylococcus aureus*. If characteristic colonies are present, perform coagulase test as follows. Transfer representative colonies to separate tubes containing 0.5 mL of rabbit plasma, horse plasma, or any other mammalian plasma. Incubate in a water bath at 37°. Examine for coagulation after 3 hours of incubation and at suitable intervals up to 24 hours. Comparing with positive and negative controls, the absence of a coagulase reaction indicates the absence of *Staphylococcus aureus* in the tested article.

Table 1. Characteristics of *Staphylococcus aureus* on Specified Agar Media

Agar Medium	Colonial Morphology	Gram Stain
Vogel–Johnson	Black surrounded by yellow zone	(+), cocci
Mannitol–Salt	Yellow colonies with yellow zone	(+), cocci
Baird–Parker	Black, shiny surrounded by 2–5-mm clear zones	(+), cocci

Test for Absence of *Salmonella* Species

Incubate at 30° to 35° for 18 to 24 hours. From *FSCD*, pipet a 1-mL aliquot into 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from both incubated media onto individual surfaces of one or more of following media: *Brilliant Green Agar Medium (BG-Agar)*, *Xylose–Lysine–Desoxycholate–Agar Medium (XLDC-Agar)*, and *Hektoen Enteric Agar Medium (HE Agar)*. Cover, invert the plates, and incubate at 30° to 35° for 24 to 48 hours. Examine the inoculated plates of *BG-Agar*, *XLDC-Agar*, and/or *HE Agar*, and interpret the results with reference to *Table 2*: if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Salmonella* species. If colonies with characteristics described in *Table 2* are present, the suspect colonies are transferred to a slant of *Triple Sugar–Iron–Agar Medium (TSI)* using an inoculating wire, by first streaking the surface of the slant, and then stabbing the wire well beneath the surface. Incubate at 30° to 35° for 24 to 48 hours. If the tubes do not have red alkaline slants and yellow acid butts, with or without concomitant blackening of the butts from hydrogen sulfide production, the test specimen meets the requirement for the absence of *Salmonella* species.

Table 2. Characteristics of *Salmonella* Species on Specified Agar Media

Agar Medium	Colonial Morphology	Gram Stain
Brilliant Green	Small, transparent and colorless; or opaque, pink or white (often surrounded by pink to red zone)	(–), rods
Xylose–Lysine–Desoxycholate	Red, with or without black centers	(–), rods
Hektoen Enteric	Blue-green, with or without black centers	(–), rods

Test for Absence of *Escherichia coli*

Incubate at 30° to 35° for 24 to 48 hours. From *FSCD*, pipet a 1-mL aliquot into a container containing 10 mL of *MacConkey Broth*, mix, and incubate at 42° to 44° for 24 to 48 hours. Streak a loopful from both incubated media onto individual surfaces of *MacConkey Agar Medium (MC Agar)*, and incubate at 30° to 35° for 18 to 24 hours. Examine the inoculated *MC Agar* plate,

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and interpret the results with reference to *Table 3*: if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Escherichia coli.* Suspect colonies showing the characteristics described in *Table 3* are transferred individually, using an inoculating loop, to the surface of a plate with *Levine Eosin–Methylene Blue–Agar Medium (LEMB-Agar).* If a large number of suspect colonies are to be transferred, divide the surface of each plate into quadrants, each quadrant being inoculated with a different colony. Cover the plates, invert, and incubate at 30° to 35° for 24 to 48 hours. If none of the colonies exhibit a characteristic metallic sheen under reflected light, and if none exhibit a blue-black appearance under transmitted light, the test specimen meets the requirement for the absence of *Escherichia coli.*

Table 3. Characteristics of *Escherichia coli* on *MacConkey Agar Medium*

Colonial Morphology	Gram Stain
Brick red, may have surrounding zone of precipitated bile	(–), rods

Test for Absence of *Clostridium* Species

TEST PREPARATION

Prepare as directed for *Sampling.* [NOTE—On the basis of results for *Preparatory Testing,* modify the *Test Preparation* as appropriate.]

PROCEDURE—Take two equal portions of the *Test Preparation,* heat one to 80° for 10 minutes, and cool rapidly. Transfer 10 mL of each portion to separate containers, each containing 100 mL of *Reinforced Medium for Clostridia,* and incubate under anaerobic conditions at 35° to 37° for 48 hours. After incubation, subculture each specimen on *Columbia Agar Medium* to which gentamicin has been added, and incubate under anaerobic conditions at 35° to 37° for 48 hours. Examine the plates, and interpret with reference to *Table 4*: if no growth of microorganisms is detected, the test specimen meets the requirement for the absence of *Clostridium* species.

Table 4. Characteristics of *Clostridium* Species on Specified Media

Medium	Gram Stain	Catalase
Reinforced Medium for Clostridia	(+), rods	
Columbia Agar	(+), rods	Negative

If growth occurs, subculture each distinct colony on *Columbia Agar Medium,* and separately incubate in aerobic and in anaerobic conditions at 35° to 37° for 48 hours. The occurrence of only anaerobic growth of gram-positive bacilli, giving a negative catalase reaction, indicates the presence of *Clostridium sporogenes.* To perform the catalase test, transfer discrete colonies to glass slides, and apply a drop of dilute hydrogen peroxide solution: the reaction is negative if no gas bubbles evolve. If the test specimen exhibits none of these characteristics, it meets the requirement for the absence of *Clostridium* species.

RETEST

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10 g specimen, a retest on a 25 g specimen of the nutritional or dietary supplement may be conducted. Proceed as directed under *Procedure,* but make allowances for the larger specimen size.

⟨2023⟩ MICROBIOLOGICAL ATTRIBUTES OF NONSTERILE NUTRITIONAL AND DIETARY SUPPLEMENTS

INTRODUCTION

The raw materials, pharmaceutical ingredients, and active ingredients used in the manufacture of nutritional and dietary articles may range from chemically synthesized vitamins to plant extracts and animal byproducts, and these ingredients are typically not sterile. Considerable experience has accrued with these highly refined plant- and animal-derived pharmaceutical ingredients, such as microcrystalline cellulose, modified starch, lactose, and magnesium stearate, and their microbiological attributes are well established. Botanicals may be microbiologically contaminated at any point during cultivation, harvesting, processing, packing, and distribution. Major sources of microbial contamination are associated with human or animal feces used as plant manure; contaminated irrigation water and/or process water; and poor worker hygiene and sanitation practices during harvesting, sorting, processing, packaging, and transportation. Furthermore, it is essential that microbiological contamination be minimized during the manufacture of nonsterile dietary supplements. To achieve this, Good Manufacturing Practices are employed and adequate microbiological specifications are established.

Microbiological process control, control of the bioburden of raw materials, and control of the manufacturing process to minimize cross-contamination are necessary to guarantee acceptable microbial quality in the final dosage forms. Because nonaqueous or dry dosage forms do not support microbial growth because of low water activity, the microbial quality of such articles is a function of the microorganisms introduced through ingredients or during processing. In addition to considering the intended use of the product, the frequency of microbial testing for the finished nonsterile dietary supplement would be a

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function of the historical microbial testing database of that product, knowledge of the manufacturing processes, the susceptibility of the formulation to microbial proliferation, and the demonstrated effectiveness of programs controlling the raw materials.

FORMULATION AND PROCESS DESIGN

From a microbiological perspective, the development of the formulation of nutritional or dietary supplements includes an evaluation of raw materials and their suppliers and the contribution made to the products by each ingredient and the manufacturing processes. Characterization of these elements allows the adequacy of the manufacturing process to be demonstrated. For example, if a product is formulated with an ingredient of botanical or animal origin known to possess a high, variable, or unpredictable level of microbiological contamination, it is necessary to ensure that the microbiological monitoring identifies ingredients that have an inappropriate bioburden level and that a premanufacturing process such as drying, extraction, heat treatment, irradiation, or gaseous sterilization treatment will inactivate or remove any objectionable contaminant possibly present.

However, the selected treatment technique should not have any adverse effects. The treatment of raw materials by irradiation and ethylene oxide may cause unwanted changes affecting the safety and efficacy of the raw material. For instance, when treated by ethylene oxide, crude extracts containing alkaloids have shown reduced contents of alkaloids. Dry heat treatment has been used for inactivation as well, but requires further evaluation because it may adversely affect stability and degradation of the raw material. With regard to the design of the manufacturing process, appropriate consideration should be given to the microbiological effect of wet granulation manufacturing processes. Wetting of a dry powder can result in increased levels of microorganisms if the granulation is stored prior to drying. However, it is recognized that the pressure and temperature associated with compression of tablets will decrease microbial counts. Antimicrobial activity is also achieved, especially with aqueous preparations, by the addition of chemicals that have known antimicrobial properties and that are compatible with the formulation.

However, antimicrobial preservation is not a substitute for Good Manufacturing Practices. A process has to be designed to minimize the microbiological population. Operating procedures, temperatures, and time limits, including holding times, are established to protect the product from microbiological contamination and growth. All processes have to be validated for their intended purposes. Moreover, in-process manufacturing and testing controls necessary for microbiological quality should be identified and implemented.

FACILITIES, EQUIPMENT, WATER, AND SANITIZATION

Facilities

The facilities, including the building and the heating, ventilation, and air-conditioning (HVAC) systems, should be designed to minimize microbiological contamination. The design of facilities used for the manufacture of supplements and their operating parameters should be documented, and the documentation should include, when appropriate, HVAC filter types, space pressure differentials, temperature, and relative humidity and air changes. Dry products processed in a dry environment do not possess a high potential for increased microbial levels. However, some control is warranted to minimize microbiological and chemical contamination. Potentially problematic areas are those that utilize *Purified Water* for wet granulation, batching liquid products, and film-coating tablets, because water encourages microbial growth.

Equipment

Equipment used for the processing of semisolid and dry supplements should be designed to promote sanitary conditions, to be self-drying, and to be easy to clean. Dryers, ovens, wet granulation equipment, bulk tanks, and equipment for preparation of coating solutions are periodically evaluated to ensure that cleaning procedures are adequate.

Water

As one of the major components in nutritional and dietary supplement manufacturing processes, water deserves a special consideration in the microbiological control of these articles. It is a growth medium for a variety of microorganisms that present a threat to product quality, safety, preservation, and stability. Water may even act as a carrier of objectionable microorganisms. In view of this, water used in manufacturing is *Purified Water*. For the manufacture of raw materials, process water that meets specific microbiological objectives and U.S. Environmental Protection Agency National Drinking Water standards or equivalent European and Japanese standards may be used.

Cleaning and Sanitization

Detailed and specific cleaning and sanitization procedures should be evaluated, developed, and validated, with special attention given to product contact surfaces. Personnel should possess sufficient knowledge of these procedures.

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SUPPLEMENT COMPONENTS

Raw materials, excipients, and active substances as components of nutritional and dietary supplements can be a primary source of microbiological contamination. Specifications should be developed and sampling plans and test procedures should be employed to guarantee the desired microbiological attributes of these materials. The nature and extent of microbiological testing should be based upon a knowledge of the material's origin, its manufacturing process, use, historical data, and experience. For instance, materials of animal or botanical origin that are not highly refined might require special, more frequent testing than synthetic products.

Since members of the family Enterobacteriaceae are a major component of the normal epiphytic and endophytic microflora (e.g., members of genera *Klebsiella, Enterobacter,* and *Erwinia*) and have been associated with the seeds, pods, roots, leaves, and stems of plants of economic importance, coliform or Enterobacteriaceae counts will not be an appropriate general microbiological criterion for botanicals. However, when it is considered advantageous, coliform or Enterobacteriaceae counts may be included in the individual monographs. Typically on new leaves, bacteria predominate in the microflora, while yeast and filamentous fungi succeed bacteria and become dominant late in the growing season. With dried botanicals, the bacterial population will tend to change from Gram-negative bacteria to Gram-positive spore formers and fungi. Refinement of botanicals from chopped or powdered plant material to powdered extracts using alcoholic, alkaline, acid hydro-alcoholic, or aqueous extracting materials will reduce the likelihood of vegetative microorganisms within the botanical material. The classification of botanical materials is contained in *Table 1*.

Table 1. Definitions of a Range of Botanical Materials

Botanical Preparation	Definition
Chopped or Powdered Botanicals	Hand-picked portions of the botanical (e.g., leaves, flowers, roots, tubers, etc.) that are air dried, chopped, flaked, sectioned, ground, or pulverized to the consistency of a powder.
Botanical Extracts	Extracts are solids or semisolid preparations of a botanical that are prepared by percolation, filtration, and concentration by evaporation of the percolate. The extracting material may be alcoholic, alkaline, acid hydro-alcoholic, or aqueous in nature. Typically, an extract is 4–10 times as strong as the original botanical. The extracts may be semisolids or dry powders termed powdered extracts.
Tinctures	Tinctures are solutions of botanical substances in alcohol obtained by extraction of the powdered, flaked, or sectioned botanical.
Infusions	Infusions are solutions of botanical principles obtained by soaking the powdered botanical in hot or cold water for a specified time and straining. Typically, infusions are 5% in strength.
Decoctions	Decoctions are solutions of botanicals prepared by boiling the material in water for at least 15 min and straining. Typically, decoctions are 5% in strength.
Fluidextracts	A fluidextract is an alcoholic liquid extract made by percolation of a botanical so that 1 mL of the fluidextract represents 1 g of the botanical.
Botanicals to be treated with boiling water before use	Dried botanicals to which boiling water is added immediately prior to consumption.

MICROBIOLOGICAL TESTING

Frequency of Sampling and Testing

Microbiological attribute sampling and testing plans vary widely. In some cases, no sampling or testing is necessary; in other cases, periodic monitoring is warranted; and yet for some articles, each batch requires sampling and testing. The design of the sampling and testing plans and the kind of attributes examined depend on the application and the type of the product, the potential for contamination from components and processing, the growth promotion or inhibition properties of the formulation, and the target population for the supplement. For example, a powdered botanical may have highly variable microbiological attributes so that an incoming batch would be sampled and composite testing would not be advised, while a highly refined botanical extract may not require routine microbial testing. Similarly, products with a low water activity will not be susceptible to microbial growth during their shelf life provided they are protected from elevated humidity by their containers.

Microbial Enumeration Tests

See the *Introduction* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩. These tests provide meaningful information regarding the microbiological acceptability of excipients, active substances, and nonsterile supplement formulations. If the individual monograph does not specify microbial enumeration limits, the guidance provided in this chapter is used. Acceptable general limits of microbial levels for raw materials, excipients, and botanical products are shown in *Table 2*; and those for raw materials, excipients, active ingredients, and other nonsterile finished articles that are nutritional supplements, but do not contain botanicals, are shown in *Table 3*.

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Table 2. Recommended Microbial Limits for Botanical Ingredients and Products

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Dried or Powdered Botanicals	Total aerobic microbial count NMT 10^5
	Total combined yeasts and molds count NMT 10^3
	Bile-tolerant Gram-negative bacteria NMT 10^3
	Absence of *Salmonella* spp. and *E. coli* in 10 g
Powdered Botanical Extracts	Total aerobic microbial count NMT 10^4
	Total combined yeasts and molds count NMT 10^3
	Absence of *Salmonella* spp. and *E. coli* in 10 g
Tinctures	Total aerobic microbial count NMT 10^4
	Total combined yeasts and molds count NMT 10^3
Fluidextracts	Total aerobic microbial count NMT 10^4
	Total combined yeasts and molds count NMT 10^3
Infusions/Decoctions	Total aerobic microbial count NMT 10^2
	Total combined yeasts and molds count NMT 10
Nutritional Supplements with Botanicals	Total aerobic microbial count NMT 10^4
	Total combined yeasts and molds count NMT 10^3
	Absence of *Salmonella* spp. and *E. coli* in 10 g
Botanicals to be treated with boiling water before use	Total aerobic microbial count NMT 10^6
	Total combined yeasts and molds count NMT 10^4
	Bile-tolerant Gram-negative bacteria NMT 10^2
	Absence of *E. coli* and *Salmonella* spp. in 10 g

Table 3. Recommended Microbial Limits for Dietary Supplement Ingredients and Products

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Other raw materials and dietary supplement ingredients	Total aerobic microbial count NMT 10^3
	Total combined yeasts and molds count NMT 10^2
	Absence of *E. coli* in 10 g
Nutritional supplements with synthetic or highly refined ingredients	Total aerobic microbial count NMT 10^3
	Total combined yeasts and molds count NMT 10^2
	Absence of *E. coli* in 10 g

Absence of Objectionable Microorganisms

See *Introduction* under *Microbiological Procedures for Absence of Specified Microorganisms—Nutritional and Dietary Supplements* ⟨2022⟩. Absence of one or more species of objectionable microorganisms is required in some individual monographs.

Test for Aflatoxins

Dietary and nutritional articles containing botanical products with a history of mycotoxin contamination are also typically tested for aflatoxins, especially if the material is obtained from roots or rhizomes. See *Articles of Botanical Origin* ⟨561⟩ for the details of a test for aflatoxins. Where necessary, this test is included in the individual monograph.

Solid Oral Dosage Forms

Among all dosage forms, solid oral dosage forms present the lowest microbiological risk because of their method of manufacture, low water activity, and route of administration. When justified, reduced microbiological testing may be appropriate.

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Other Concerns

The presence of some microorganisms in articles can be an indicator of processes that are not under microbiological control. For example, *Purified Water* used at some stage of the manufacture of these products might contain a typical flora of Gram-negative microorganisms. As with pharmaceutical products, inadequate processing of water and poor maintenance of water systems may result in the contamination of processed formulations by Gram-negative microorganisms.

⟨2030⟩ SUPPLEMENTAL INFORMATION FOR ARTICLES OF BOTANICAL ORIGIN

This general chapter provides information about several aspects of botanical articles not covered in *USP* standards monographs. Although the standards in the monographs address the quality issues associated with botanical plant materials, extracts, and preparations of Pharmacopeial articles, there is a need to develop appropriate information to optimize the pre-harvest conditions for appropriate growth and the post-harvest handling to achieve consistent quality with minimum variations in the composition of chemical constituents.

PROTOCOL CONTENTS

Black Cohosh (*Actaea racemosa* L.)
Ginger (*Zingiber officinale* Roscoe)
Valerian (*Valeriana officinalis* L.)
Elm (*Ulmus rubra* Muhlenberg)

GENERAL GUIDANCES

It is recommended that, at a minimum, growers and others involved in the handling and distribution of botanical products should become familiar with and follow the WHO Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants (found at http://apps.who.int/medicinedocs/pdf/s4928e/s4928e.pdf).

Commercial trade in natural products occurs in a global market. Material of domestic origin must be produced in compliance with all federal laws of the United States. Material of foreign origin, imported into the U.S., must be produced and transported in compliance with the laws of the U.S., the country of origin, and relevant international treaties. These include, but may not be limited to, the following:

1. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments. Its aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival. Information about CITES is available at http://www.cites.org.
2. The Convention on Biological Diversity (CBD) establishes three main goals: the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits from the use of genetic resources. Each country that has ratified and is a party to the Convention is responsible for implementation by means of national enabling legislation that can differ from country to country.
3. The Endangered Species Act (ESA) was originally adopted in 1973. The ESA is a law that aims to protect species of fish, wildlife, and plants believed to be threatened with extinction. The ESA is administered primarily by the U.S. Fish and Wildlife Service. Full text of the act is available at: http://epw.senate.gov/esa73.pdf.

Provided below is additional information not covered in the compendial specifications: compendial history; sources; collection and cultivation, including common adulterants; and drying, storing, and shipping. This information is provided to complement the standards for quality control in the monographs for botanical articles.

Compendial History—The focus in this section is on historical compendial use that has strong validity, with only brief reference to anecdotal use. This is important information because traditional use is one of the elements taken into consideration to support the safety and the presumptions of benefits of botanical dietary supplements.

Sources—Included here is the point of origin of the botanical; it also encompasses cultivation (defined as agricultural growing) and wildcrafting (defined as collected in the wild), along with a listing of the primary geographical (native) areas of production.

Collection and Cultivation—This section discusses wildcrafting, the conservation of restricted and rare species, and the trend to cultivation as an ecological alternative; such optimal harvesting and collection practices serve to preserve the integrity of species and botanical products. It is divided into three subsections:

1. Collection (conservation and ecology)
2. Cultivation Practices
3. Optimal Times for Harvest

Post-Harvest Handling (Optimal Handling and Processing Practices, Drying, Storage, and Shipping)—Important factors regarding storage of herbal products and how they should be maintained include the following.

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1. *Light:* Protection from light is important for botanical articles. Light accelerates numerous chemical processes that may lead to degradation or changes in the constituents of the articles.
2. *Temperature:* Storage temperatures in this *Pharmacopeia* are defined in *Packaging and Storage Requirements* ⟨659⟩, *Storage Conditions*. Excessive heat may affect the content of volatile constituents (essential oils) and accelerate degradation processes. However, heat treatments are sometimes useful in the maintenance of the article's quality and can be used in drying, reducing microbial load, and inhibiting certain enzymes. Heat application during these processes must be carefully controlled to achieve the desired balance between degradation and quality conservation.
3. *Humidity:* Moisture in the articles may allow certain enzymes such as glycosidases to become active, hence degrading constituents. High humidity also increases the danger of microbial proliferation. As a rule, it is advisable to store botanical articles below 60% relative humidity. Although controlled humidity and temperature warehouses are now required in many good manufacturing practices for natural products, much of the world still lacks access to these facilities.
4. *Degree of Comminution:* The degree of comminution plays a role in determining the stability of the botanical articles during storage. The increased surface area in fine powders allows oxidation and other degradation processes to occur more extensively and rapidly than in the case of a whole article. Plants containing tannins, bitter substances, and essential oils are particularly sensitive to the degree of comminution. In general, dried crude botanicals should be stored in a minimally processed form.
5. *Containers:* Appropriate containers are defined in this *Pharmacopeia* in *Packaging and Storage Requirements* ⟨659⟩, *General Definitions*.

Constituents—Where known, the substances mainly responsible for the activity of the product are listed, along with other compounds contained in the plant.

SUPPLEMENTAL INFORMATION AND GENERAL GUIDANCE PROTOCOLS

Black Cohosh *Actaea racemosa* L.[*Cimicifuga racemosa* (L.) Nutt.] (Fam. Ranunculaceae)

BOTANICAL IDENTIFICATION

Actaea racemosa L. Herbaceous perennial from rhizome.
Stem: Erect, solitary, to 2.5 m tall, glabrous.
Leaves: Basal and cauline, alternate, 2–4-ternately compound, petioles 15 to 60 cm long, bases clasping stem; leaflets 20 to 70; terminal leaflet of central division 3-lobed, 6 to 15 cm long, 6 to 16.5 cm wide, with 3 prominent veins arising from base; subterminal leaflets with blades ovate-lanceolate to obovate, 4 to 12 cm long and 3 to 8 cm wide; margins toothed to deeply incised; green above, paler below; glabrous or rarely pubescent along veins of undersurface.
Inflorescence: Terminal panicle of 4 to 9 slender branches, each 7 to 60 cm long, pubescent; 1 bract subtending each pedicel.
Flowers: Perfect, radially symmetric; sepals 4, greenish-white, caducous; petals 0; staminodes (1–) 4 (–8), petaloid, cream-colored, 2 to 3 mm long, clawed, apex bifid; stamens 55 to 110; pistils 1 (–3), glabrous to pubescent, ovary superior, style short, stigma 0.5 mm wide.
Fruit: Many-seeded follicle, 5 to 10 mm long, ovoid, laterally compressed with curved, stout beak (peristent style), pubescent; seeds hemispheric, brown, scales lacking.
Chromosome number: n = 8.

There are currently two varieties of *A. racemosa* recognized on the basis of differences in leaf morphology: var. *racemosa* and var. *dissecta*. The former variety has triternate-pinnate leaves with serrate margins, while the latter has quadriternate-pinnate leaves that are deeply incised with serrate lobes. Variety *dissecta* is only known from very few herbarium specimens, all of which were collected well over 100 years ago, making this taxon of uncertain taxonomic significance.

COMPENDIAL HISTORY

Black cohosh appeared on the secondary list of substances in the first *United States Pharmacopoeia (USP)* of 1820, where it was listed as an anti-inflammatory and antispasmodic. It soon rose to the primary list in 1830, a position it held until the 10th decennial revision of 1920. Black cohosh appeared in the first edition of the *United States Dispensatory (USD)* in 1833 and remained through 1955 for a total of 122 years. Carrying forward the traditional Native American use of black cohosh for women's ailments and Barton's use for throat complaints, current therapeutics finds the plant used in a number of preparations for coughs and for gynecological disorders. In 2001, both the rhizome and the dry rhizome extract of black cohosh were proposed once again for inclusion in the *United States Pharmacopeia–National Formulary (USP–NF)*. (See revised proposal on page 1455 of *PF* 28(5) [Sept.–Oct. 2002].) The monograph became official in the *Second Supplement* to *USP 30–NF 25*.

CONSTITUENTS

Major constituents of black cohosh are triterpene glycosides principally as beta-xylopyranosides and alpha-arabinopyranosides. The aglycones are mostly derived from acteol and cimigenol. The nomenclature of these compounds is quite confusing in the literature, with different names often given to the same compounds. A cyclopropane ring is a common feature of these compounds, which are structurally related to cycloartenol. The isoflavone formononetin has been reported in some publications; however, recent evidence indicates its absence in the roots and rhizomes of *Actaea racemosa*. Other constituents include tannins, resin, fatty acids, starch, sugars, and aromatic acids including ferulic acid, isoferulic acid, caffeic acid, and salicylic acid.

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SOURCES AND DISTRIBUTION

Sources—Black cohosh can be found in moist deciduous forests, ravines, moist meadows, creek margins, and mountainous terrain. Black cohosh flowers from June to September and is native to eastern North America from Ontario south to Georgia and west to Missouri. The entire supply of black cohosh comes from the United States. The major producers of black cohosh are Kentucky and Tennessee, with additional supplies coming from Georgia, Michigan, North Carolina, Ohio, South Carolina, Virginia, West Virginia, and Wisconsin. Although there are reports of black cohosh being grown in China and India for export, the true identity of the cultivated material has not been verified and may well be an Asian species of *Actaea* such as *A. cimicifuga* (syn. *Cimicifuga foetida*). The vast majority of the commercial black cohosh is wild harvested. Concern over the conservation of black cohosh due to increasing demand makes this species a good candidate for cultivation.
Distribution—North America (Ontario; Georgia, Kentucky, Michigan, Missouri, North Carolina, Ohio, South Carolina, Tennessee, Virginia, West Virginia, and Wisconsin); China; and India.

COLLECTION AND CULTIVATION

Collection (Conservation and Ecology)—Traditionally, black cohosh has been harvested after plants become reproductive, which occurs anywhere from 2 to 8 years of age in cultivated plants, depending on growing techniques (see *Cultivation Practices*). A portion of the rhizome with a visible bud on it should be left in the ground to resprout the following year. There is no published information on the relationship between the constituent profile of the rhizome and its age, growing conditions, or place of origin, although such studies are underway. The impact of harvest on wild populations of black cohosh is currently unknown, and sources differ in their opinion about it. Whereas some maintain that current levels of harvest threaten the viability of wild populations, others feel that sustainable harvesting is possible at current levels of demand. A study of sustainable harvest limits is currently underway. The regulatory status regarding the trade of black cohosh is under review by CITES. Refraining from harvesting plants until after they have set seed and leaving a portion of the rhizome in the ground to resprout are key components to sustainable harvesting.
Cultivation Practices—Black cohosh is grown from rhizome cuttings or seeds and requires some shading, depending on altitude and other environmental conditions. If grown from rhizome cuttings, a plant takes 2 to 3 years to become reproductive; grown from seed sown in the greenhouse and then planted, takes 4 to 6 years; direct-seeded may take from 6 to 8 years. Preliminary work indicates that black cohosh can be propagated successfully using in vitro techniques.
Optimal Times for Harvest—Rhizomes and roots should be harvested in autumn when the plant is dormant. At that time the underground portions of the plant have lower moisture content than in other seasons. Fall harvesting also allows plants to produce mature seeds before being uprooted.

POST-HARVEST HANDLING

Optimal Handling and Processing Practices—Rhizomes with roots may be processed fresh or dried. They should be thoroughly washed directly after harvest and then laid out to dry. Freshly harvested roots should be solid but not woody.
Drying—Rhizomes with roots are cut and air-dried at 35° to 45°. They are fully dried when they are brittle and snap easily and when no moisture is evident in cross section, either visibly or to the touch.
Storage—Follow general guidelines for storage by packing in airtight containers protected from light, heat, moisture, and insect infestation.

ADULTERANTS AND CONTAMINANTS

Other species of *Actaea,* especially yellow cohosh (*A. podocarpa* syn. *Cimicifuga americana*), have commonly been mixed with *A. racemosa* because of similarity in aboveground appearance and common growing habitat between species. The two species can be distinguished by differences in their freshly harvested underground parts: the fresh rhizome of *A. podocarpa* has a distinct yellowish hue, whereas that of *A. racemosa* is black. The rhizomes of both species are far more difficult to tell apart when dry because *A. podocarpa* darkens upon drying. The underground portions of baneberry *(Actaea pachypoda* and *A. rubra)* occur as occasional adulterants of black cohosh supplies. Fruiting plants of baneberry may be distinguished from black cohosh by their fleshy white or red poisonous berries, which contrast with the dry follicles of black cohosh. No information was available on how to distinguish the underground portions of black cohosh and baneberry from each other. According to one herb dealer, the roots of baneberry are smaller than those of black cohosh, and therefore are not often harvested by wildcrafters. In the Pacific Northwest, *Actaea elata* (syn. *Cimicifuga elata*) is collected for medicinal use.

Ginger *Zingiber officinale* Roscoe (Fam. Zingiberaceae)

BOTANICAL IDENTIFICATION

Zingiber officinale Roscoe. Herbaceous perennial from tuberous rhizome, aromatic because of the presence of volatile oils.
Stem: Erect, unbranched pseudostem formed by the tight overlap of sheathing leaf bases; 0.9 to 1.5 m tall.
Leaf: Simple, alternate and two-ranked, sessile or petioles short with bases sheathing the stem and a ligule where the leaf base meets the stem; blade linear to narrowly lanceolate, 15 to 25 cm long, 1.5 to 3 cm wide; margin entire; glabrous to pubescent.
Inflorescence: Terminal spike, 3.5 to 8 cm long, 1.5 to 2 cm wide, with conspicuous spirally arranged primary bracts; usually borne on specialized leafless stems.
Flower: Perfect, bilaterally symmetric; calyx tubular with 3 lobes; corolla tube 2 to 2.5 cm long with lanceolate apical lobes, 1.5 to 2 cm long, 2 to 3.5 mm wide, greenish yellow; stamen 1, anther cream-colored with dark purple, elongated connective grasping upper part of style; staminodes 4, petaloid, 2 fused into an erect, ovate-oblong lip that is dull purple with cream mottling; ovary inferior; style 1, slender, exerted beyond connective.

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Fruit: Loculicidal capsule; seeds shiny black with a white aril.
Chromosome number: n = 11.

There are several different varieties and forms of ginger. The varying morphological characteristics of these are displayed in *Table 1*.

Table 1. Morphological and Key Characteristics of Ginger from Different Areas of Production

Source	Form	Aroma	Color (External)
Africa	Flat surfaces, mostly peeled, starchy and fibrous; 9 cm long, 1.5 cm wide	Poor quality is recognized by its camphoraceous aroma	Uncut surface dark grayish-brown; cut surface brownish-black
Australia		Citrus-like	Buff
Bengal	Flat surfaces, scraped		Gray-brown
China	Short stumpy lobes, unscraped, mostly sliced	Strong, floral to citrus	Pale brown
Cochin	Lateral surfaces lacking cork	Strong, floral to citrus	Cream color with numerous black resin dots
Jamaica (unbleached)	Up to 12 cm long, 1 cm wide; surfaces completely peeled; starchy and fibrous thin cortex	Delicate, citrus-like	All surfaces yellow-brown
Japan	Up to 7 cm long, 12 mm wide; flat surfaces usually completely peeled; starchy and fibrous thick cortex	Bergamot-like	Externally gray-white to light grayish-brown, often with white powder from being coated with lime
Malabar (Cochin and Calcutta)	Cork layer completely removed, mostly treated with chalk	Citrus-like	Almost white
Nigeria	Smaller in size than other varieties, rather less deeply scraped	Delicate	Somewhat darker than other varieties

COMPENDIAL HISTORY

Ginger was official in the *United States Pharmacopoeia* from the first edition of 1820 through the fourteenth revision of 1950, often appearing in multiple preparations. It also appeared in all editions of the *United States Dispensatory* from 1833 through the final edition of 1973, where it was described as "a stimulant and carminative that has been used for treatment of dispepsia and flatulent colic".

CONSTITUENTS

The essential oils and the pungent principles make up some of the major components of the rhizome of ginger: 4.0% to 10.0% of the rhizome consists of an oleoresin composed of nonvolatile, pungent principles (phenols such as gingerols and their related dehydration products, shogaols); nonpungent fats and their waxes. The essential oil (1% to 3%) contains sesquiterpenes and monoterpenes, mainly geranial and nerals. Generally, but not always, sesquiterpenes predominate (30% to 70%), such as zingiberene, sesquiphellandrene, and beta-bisabolene, which decompose on drying and storage. The nonvolatile pungent principles include the phenylalkanones, the gingerols, and the phenylalkanonols, shogaols with varying chain lengths.

SOURCES AND DISTRIBUTION

Sources—Ginger is cultivated in most tropical and subtropical countries to greater or lesser degrees. The world production is estimated to be 100,000 tons. China and India are reported to be the primary areas of production. Approximately 5000 tons of ginger are imported into the United States. An estimated 80% of this comes from China. In China, Sichuan and Guizhou provinces reportedly produce the largest quantities and highest quality. It is also produced in Guangdong, Hubei, Shandong, Shanxi, and Zhejian provinces. Most of the dried ginger from China available in the United States has had the cortex scraped or rubbed off before it is dried. This gives it a whitish appearance. The freshly dug root is soaked overnight in water, scraped with a knife to remove the outer cortex, and then sun-dried. It has been reported that high arsenic levels in the soil of Changning County of Hunan Province, China, has negatively affected ginger yields.

In India, ginger is grown on a large scale in the warm, moist regions of Madras and Cochin, and to a lesser extent in Bengal and the Punjab. Varieties grown in Bengal are reportedly the highest quality material in India. Other areas of production include Africa (Nigeria and Sierra Leone), Australia, the East Indies, Fiji, Hawaii, and Jamaica. The morphological characteristics of ginger cultivated in these different areas are outlined in *Table 1*.

In older literature, Jamaican ginger is reported to be the highest quality and the most aromatic, though supplies are limited.
Distribution—Most tropical and subtropical countries, such as Australia, China (Guangdong, Guizhou, Hubei, Shandong, Shanxi, Sichuan, and Zhejian provinces), India (Bengal, Cochin, Malabar), the East Indies, Fiji, Jamaica, Japan, Nigeria, and Sierra Leone. Hawaii in the United States.

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COLLECTION AND CULTIVATION

Collection (Conservation and Ecology)—When the stems wither and are white, the rhizomes are ready for collection. Usually ginger is harvested after 6 months of growth at the earliest, and sometimes not until as late as 20 months; or to obtain larger roots, it is harvested in January or February of the second year of growth. In tropical and subtropical areas, roots are harvested as early as 4 months of growth, because they tend to become fibrous and tough as they get older. As ginger matures, it becomes more fibrous and stronger in flavor. Ginger harvest can be described in three stages:

1. Ginger that has been harvested early is known as green ginger and is traded as fresh ginger. It is succulent and tender, mellow, and mildly aromatic with a floral or lemony aroma and mild flavor.
2. Ginger harvested a few months later is more fibrous and drier and is collected for drying and may be sold as a full-flavored, pungent dried whole ginger.
3. The last harvest is usually around 9 months and yields the strongest ginger, which is quite dry and also richest in pungent components. This ginger is dried and then ground into powder.

Cultivation Practices—Ginger is a perennial herb that grows well at subtropical temperatures where the rainfall is at least 1.98 meters per year. The plant is sterile and is grown by vegetative means. Selected pieces of rhizome ("seed pieces" or "setts"), each bearing a bud, are planted in holes or trenches. Ideally the soil should be well-drained, rich clay loam. The growing conditions resemble those of potato cultivation. Mulching or manuring is necessary because the plant rapidly exhausts the soil of nutrients.

Ginger is susceptible to waterlogging and root rot. Preventive methods include using only the cleanest ginger for planting and washing it with fungicide before planting. A study growing ginger hydroponically yielded up to 125 tons per hectare in 6 to 7 months compared to 35 tons per hectare when grown in soil.

Optimal Times for Harvest: typically in December or January.

POST-HARVEST HANDLING

Optimal Handling and Processing Practices—After harvesting, the rhizome is cleaned and stripped of its stems and roots. Each area processes its ginger differently after harvest. This results in the different quality and commercial grades available on the market. Green ginger consists of the rhizomes sent to market without drying. Unscraped or partially scraped varieties are traded as coated or black ginger. These roots have been scalded with boiling water and dried quickly. When dry, black ginger breaks with a horny, blackish, somewhat diaphanous fracture, due to the pasty condition of the starch. White ginger is bleached, usually by rubbing with chalk or lime, to lighten its color and to prevent insect infestation. Preserved ginger consists of soft, yellowish-brown pieces obtained by steeping the fresh ginger in hot syrup and carefully bottling. It is soft, brown-yellow and translucent. When baked, ginger loses its pungency and acquires a bitter taste.

Drying—In general, after harvest, the fresh roots are washed, and the whole dark outer skin, consisting of cork and a little underlying parenchyma, is scraped away. Scraping speeds up the drying time of the crude drug. However, excessive scraping can result in lower concentrations of essential oil that is lost with the discarded epidermal tissue. After scraping, the rhizomes are then laid out on clean floors and dried in the sun for 7 to 10 days. During this time they are occasionally turned and are piled up every night. If the fresh rhizomes are too fleshy or moist, drying will take longer and the product will end up looking shriveled. To obtain a whiter product, the ginger is moistened after 5 or 6 days and dried for another 2 days, at which time it is ready for export. Dried ginger is more pungent and stronger in taste than fresh ginger.

Storage—Store in a tightly closed container, protected from light and moisture, in a cool area. A study was done on ginger harvested after 8, 9.5, 11, or 12 months. Samples were stored at 10° to 15° and 45% to 55% relative humidity or 25° to 30° and 75% relative humidity for 0, 4, or 8 weeks. Oil and oleoresin yields increased with the age of the ginger. Room temperature storage had adverse effects, but refrigerated storage for up to 4 weeks had no effect on quality. When stored for extended periods of time, ground ginger loses its pungency.

ADULTERANTS

Because ginger is so characteristic, unintentional adulterants are rare. However, in East Asia sometimes the much larger *Zedoary cassumer* and *Zedoary zerumbet*, along with *Alpinia allughas*, are used and found in European commerce. They are easy to distinguish because of their characteristic aromas. Occasionally, Chinese sugar-candied "ginger" is prepared from *Alpinia galangal*.

In older literature, other herbs have reportedly been used as adulterants. These include various species of *Curcuma*, *Capsicum*, and Grains of Paradise *(Amomum melegueta)* added to exhausted material in order to enhance color and pungency.

Ginger powder is sometimes adulterated with plant starches such as those from wheat middlings, potatoes, corn, barley, rice, legumes, acorns, flaxseed meal, mannihot, oil cakes from linseed, rapeseed, mustard, almond meal, palm kernel or olives, hazelnut shells, and mineral additives. These may be easily identified microscopically. The extent of this type of adulteration in trade is unknown.

Exhausted material should be considered an adulterant.

Valerian *Valeriana officinalis* L. (Fam. Valerianaceae)

BOTANICAL IDENTIFICATION

Valeriana officinalis L. Herbacious perennial, rhizomatous.

Stem: Solitary, hollow, 15 to 150 cm.

Leaf: Basal and cauline, opposite, oddly once pinnately lobed, lobes 11 to 21 lanceolate, entire or dentate, basal leaves petiolate, cauline leaves subsessile to clasping.

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Inflorescence: Compound cyme, terminal or axillary, many pale pink to white, strongly scented flowers.
Flower: Calyx 5-lobed, lobes inconspicuous in flower, becoming elongate and pappus-like in fruit, corolla funnel-form, slightly saccate at the base, 5-lobed, tube 4 mm, lobes 1 mm, stamens 3, filaments attached to corolla tube alternate to corolla lobes, ovary inferior, triloculate, uniovulate, only 1 locule fertile, stigma tripartite.
Fruit: Achene crowned by persistent calyx, lanceolate-oblong, 4.5 to 5 mm, hairy or glabrous. Populations of *V. officinalis* range in ploidy level from diploid to tetraploid or octaploid. British *V. officinalis* is usually octaploid, and central European supplies are tetraploid.

There are three subspecies of *V. officinalis*: ssp. *officinalis,* ssp. *collina* (Wallr.) Nyman, and ssp. *sambucifolia* (Mikan fil.) Celak. All three of these subspecies, as well as the other European species of valerian, *V. repens* Host, have been considered acceptable source material for medicinal preparations.
Macroscopic Identification—Various chemotypes will have slightly different characteristics. When dried, the whole rhizome is up to 50 mm long and up to 30 mm in diameter, obconical to cylindrical, with an elongated or compressed base. It has a yellowish-brown to dark brown exterior with a circular stem and leaf scars. The rhizome contains numerous thick, light to dark brown rootlets that are located around a thin ligneous cord. The root is longitudinally wrinkled, approximately 100 mm long and 1 to 3 mm in diameter, almost cylindrical, and almost the same color as the rhizome. In longitudinal section, the pith exhibits a central cavity transversed by septa. The stolons are 20 to 50 mm long, pale yellowish grey with prominent nodes separated by longitudinally striated internodes. It is commonly sliced in half for ease of cleaning. The rootlets, which contain the majority of the essential oil, are brittle and break in short, horny fractures and are whitish or yellowish internally. Aroma: when dried properly, *V. officinalis* L., s.l. has only a very faint characteristic, valeric acid–like aroma that becomes stronger as it ages. Improperly dried or old material possesses a strong and characteristic odor due to the enzymatic hydrolysis of esters of the valepotriates (isovaleric acid and hydroxyvaleric acid). Taste: mildly sweet and camphoraceous with a slightly bitter and spicy aftertaste.

COMPENDIAL HISTORY

Valerian was official in the *United States Pharmacopoeia* from the first edition of 1820 through the eleventh revison of 1930, often appearing in multiple preparations. At its peak from 1850 through 1880 it appeared six to seven times in different preparations. Valerian is among the top 30 most listed botanicals in the history of the *USP*. The root of valerian has been used as a sedative and spasmolytic in Europe since the 16th century.

CONSTITUENTS

Major constituents of valerian have been identified as sesquiterpenes of volatile oils and iridoids (epoxy-triesters) known as valepotriates. The total content of volatile oil varies widely within a single species and between different species. European *Valeriana officinalis* L. usually contains 0.1% to 2.8% volatile oil. The oil consists of mixtures of monoterpene and sesquiterpene derivatives. The amount of valepotriates present also varies widely between species and genera and even within a species, generally ranging from 0.5% to 1.2%. Valepotriates are particularly unstable; they decompose easily under the effect of moisture, temperatures above 40°, or acidity (pH <3).

Valerian also contains small amounts of aliphatic acids, alkaloids, amino acids, phenolic acids, flavonoids, free fatty acids, sugars, and salts. Valerian constituents that have possible sedative effects include acetoxyvalerenic acid, 1-acevaltrate, baldrinal, didrovaltrate, hydroxyvalerenic acid, kessane derivatives, valeranone, valerenal, valerenic acid, and valtrate.

SOURCES AND DISTRIBUTION

Sources—Valerian is found in damp or dry meadows, scrub, or woods in most of Europe, although rare in the south, and it is cultivated and naturalized in North America. Valerian is cultivated in Belgium, Britain, Eastern Europe, France, Germany, Japan, the Netherlands, North America, and Russia. The majority of standardized extract products and crude cut and sifted material on the domestic market are prepared from European supplies. A large number of liquid extracts are prepared from domestically cultivated material. Many species other than *V. officinalis* are reported to be traded as medicinal valerian. These include *V. edulis* Nutt. ex Torr. & A. Gray, *V. corneana* Briq. k, *V. stubendorfi* Kreyer ex Kom., *V. amurensis* P. Smirn. ex Kom., *V. hardwickii* Wall., *V. exaltata* Mikan, and *V. wallichi* DC. syn. *V. jatamansi* Jones.* The most frequently used North American species include *V. sitchensis* Bong and *V. edulis* Nutt.* = *V. edulis* Nutt. ex Torr. & Gray ssp. *procera.* Other species reported to be used locally include *V. arizonica* Gray, *V. capitata* Pall ex Link., *V. diocia* L., and *V. scouleri* Rydb. Detailed chemical analyses of most American species are lacking. A limited number of assays of material cultivated in the Pacific Northwest show varying levels of essential oil ranging from 0.4% to 1.3%. Valerenic acid and valepotriates have been found to be present in fresh and dry samples of *V. sitchensis* Bong. *V. sitchensis* Bong exhibits a strong pungency when fresh. High quality material is reported to contain from 1.0% to 1.5% essential oil, ≥30% extractable matter, and ≥0.5% valerenic acid.
Distribution—Europe (Belgium, Britain, Eastern Europe, France, Germany, the Netherlands), Japan, North America, and Russia.

COLLECTION AND CULTIVATION

Collection (Conservation and Ecology)—The majority of valerian in trade comes from cultivated material. Harvest times will vary geographically. The composition of the essential oil varies greatly among different populations of the same subspecies and even between the same population of plants from year to year. Essential oil content also varies with genotypes, harvest times, growing conditions, age of root, drying techniques, and method of analysis. It has been reported that valerian harvested in higher elevations, grown in dryer regions, or cultivated in phosphate-rich soil yields relatively high levels of essential oil.

* *V. wallichi* DC. and *V. edulis* Nutt. reportedly are lacking in valerenic acid and its derivatives.

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Older literature reports that valerian should be harvested in the fall, between August and September, preferably in the second year of growth. Analyses of material cultivated in the Netherlands report that the majority of constituents, including the essential oil and valerenic acid, were highest in roots harvested in the first year of growth, with essential oil being highest in September and November (1.2% to 2.1%). The next highest level of essential oil was reported for material harvested in March (0.9% to 1.6%). Valerenic acid and its derivatives were found to be highest in February and March (0.7% to 0.9%), followed by material harvested in September (0.5% to 0.7%) and then in January (0.3% to 0.4%). From a commercial standpoint, it is more cost effective to harvest the roots in the same year the plants are sown than in the second year.

Cultivation Practices—Sowing seeds has been reported to be preferred over planting of seedlings. Best results were achieved by flat field planting at row spacings of 50 cm and a seed rate of 3 kg per hectare. Cutting off the flowering tops before the plant has set seed causes the rhizome to develop more fully.

Optimal Times for Harvest—Wagner reports that harvest should take place in the morning during relatively cool weather, a general recommendation for roots rich in essential oils.

POST-HARVEST HANDLING

Optimal Handling and Processing Practices—The essential oil is located in the hypodermis of the rhizome in large thin-walled cells. Therefore, care must be taken not to damage these cells during handling. Excess washing of the roots can result in a significant reduction of extractive matter. Because of the sensitivity of volatile oils to heat, it is necessary to minimize the amount of time generated in the grinding or powdering process by doing small lots at a time, with frequent interruptions in run times, or by using a cryogenic grinder.

Drying—For maximum preservation of the essential oils, valerian should be dried at 40° with a flow rate of 0.05 kg per sec per m^2. Alternatively, drying at 20° for approximately 10 days, shade drying at approximately 45°, low temperature vacuum-drying, and freeze-drying are also reported to be appropriate drying techniques.

Careless or prolonged drying produces a darker color in the roots and results in the hydrolysis of the isovalerianic esters and the liberation of isovaleric and hydroxyisovaleric acid. This produces the characteristic valerianic aroma. Properly dried valerian will produce this same aroma over time.

Storage—Store in closed containers protected from light, air, and moisture. Hydroxyvalerenic acid, a decomposition product of acetoxyvalerenic acid, is formed when the herb is stored at too high humidity.

Improper storage conditions can cause significant deterioration of the material. Although the essential oil is relatively stable, it can evaporate with excessive exposure to air. The essential oil can degrade quickly in powdered material. In powdered root, the essential oil content can decrease by 50% within 6 months.

Valepotriates are sensitive to humidity, temperatures above 40°, and acid media (pH <3) and are generally not detected in commercial products after 60 days.

ADULTERANTS

Other species of valerian: An unidentified *Apiaceae* species may be found in valerian trade. Adulteration of valerian in the American market is not common. Many species other than *V. officinalis* are reported to be traded as medicinal valerian. These include *V. edulis* Nutt. ex Torr. & A. Gray, *V. coreana* Briq. k, *V. stubendorfi* Kreyer ex Kom., *V. amurensis* P. Smirn. ex Kom., *V. hardwickii* Wall, *V. exaltata* Mikan, and *V. wallichi* DC. syn. *V. jatamansi* Jones.

Elm *Ulmus rubra* Muhlenberg [*Ulmus fulva* Michaux] (Fam. Ulmaceae)

BOTANICAL IDENTIFICATION

Ulmus rubra Muhlenberg; tree to 35 m high, with spreading branches and open flat crown; preparations derived from inner bark. *U. rubra* appears to be more closely related to the introduced Asian species *U. pumila* L. than to other native American species of *Ulmus*; where the two co-occur, interbreeding is common.

Trunk: 18 to 35 m high, to 1 m in diameter; the trunk rises free of branches until about 5 to 6 m.

Branches: Erect, spreading; young twigs are scabrous-pubescent.

Bark: Dark brown to reddish-brown, deeply furrowed. Inner bark is whitish (outer surface yellow-orange; inner surface pale yellow), fragrant (upon powdering, a distinctive fenugreek-like odor) and very mucilaginous upon chewing or moistening.

Leaves: Alternate; simple; petiolate with petiole (3–)5–7(–9) mm long; 7–18(–23) cm long, 5–10(–15) cm broad; elliptical to ovate, oblong, or obovate with oblique base and acuminate apex; margins serrate toward base, elsewhere doubly serrate; upper surface scabrous, rough; lower surface tomentose; secondary veins parallel, slightly curved, running to tips of marginal teeth.

Inflorescence: Axillary fascicles, roughly hemispherical, to 1.5(–2.5) cm in diameter.

Flowers: Small, perfect; pedicels 1–2(–3) mm long; calyx campanulate, 5–9-lobed at apex, about 2.6–3.5 mm in diameter, reddish-pubescent; petals absent; stamens 5–9, exserted at flowering; styles 2. Flowers occur before the leaves from March through early May.

Fruit: Winged samara, yellowish, irregularly suborbicular or occasionally broadly elliptical or obovate, 10–20 mm in diameter, reddish-pubescent over seed; wing papery-textured.

COMPENDIAL HISTORY

Slippery elm (Ulmus) inner bark appeared in the list of materia medica in the first *United States Pharmacopoeia (USP)* of 1820 and remained official until it was removed from *USP XI* (1936). The *USP 1820* included instructions for the preparation of *Infusion of Slippery Elm: "Take of Slippery elm, sliced, one ounce. Boiling water, one pint. Infuse for twelve hours in a covered vessel, near the fire with frequent agitation, and strain."* Immediately following its removal from *USP XI* (official: June 1, 1936), *Slippery Elm Bark* became an official monograph in the sixth edition of the *National Formulary* (*NF*; official: June 1, 1936) until its

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elimination from the 11th edition (official: October 1, 1961). It became official again, as *Elm,* on November 15, 1995, in the *USP* section of the *Third Supplement* to the *United States Pharmacopeia–National Formulary* (*USP 23–NF 18*). A revision was published in the *Seventh Supplement* on November 15, 1997.

In 1982, Elm Bark appeared in the Food and Drug Administration (FDA) Advance Notice of Proposed Rulemaking (ANPR) for the establishment of a therapeutic monograph for oral health care drug products for over-the-counter (OTC) human use. In the ANPR (1982) as well as in the subsequent tentative final monograph of 1988 and in the amendment to the monograph of 1991, Elm bark was classified as a Category I (Generally Recognized as Safe and Effective (GRASE)) OTC oral demulcent active ingredient, and appropriate standards were urged to be developed in the official compendia.

Aside from *USP–NF,* the monograph of *Elm* had already appeared in the second edition of *The Dispensatory of the United States of America* (1834), and its last appearance was in the 25th edition, 1960.

CONSTITUENTS

Constituents of relevance for conformance to *Identification A* under *Elm* are mucilaginous substances. Elm inner bark mucilage is readily extractable by water and consists principally of a polysaccharide which on hydrolysis yields D-galactose, D-methyl galactose, L-rhamnose, and glucose. Borohydride reduction of the periodate-oxidized polysaccharide affords, on partial hydrolysis with hot acid, three oligosaccharides:
O-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-*O*-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-L-rhamnose,
O-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-L-rhamnose, and *O*-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-3-*O*-methyl-D-galactose.

Other Elm constituents are traces of tannins, including proanthocyanidins, some starch, traces of oxalate salts, beta-sitosterol, and minerals.

SOURCES AND DISTRIBUTION

Sources—Slippery elm bark is harvested from wild populations in eastern Canada and the United States, from southern Quebec west to North Dakota, south to south-central Texas, and Florida. It is common throughout eastern, southern, and midwestern U.S., and it grows in more than 25 states. An increasing amount of the commercial supply is being collected according to sustainable wild resource management plans as a condition of organic certification for wild crops. Conversely, Dutch elm disease has had a significant negative impact on elm populations, from 1930 when it was first found in the United States affecting over 50% of elm trees in the northern states.

Distribution—Canada (New Brunswick, Ontario, Quebec), the United States (Alabama, Arkansas, Connecticut, Delaware, the District of Columbia, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maine, Maryland, Massachusetts, Michigan, Minnesota, Mississippi, Missouri, New Hampshire, New Jersey, New York, North Carolina, North Dakota, Ohio, Oklahoma, Pennsylvania, Rhode Island, South Carolina, South Dakota, Tennessee, Texas, Virginia, Vermont, Wisconsin, and West Virginia).

COLLECTION AND CULTIVATION

Collection (Conservation and Ecology)—"Natural" (both inner and outer bark) and "rossed" (inner bark only) are harvested, but only the rossed bark will conform to the standards of the *USP* monograph. Harvesting should occur on dry, preferably warm days with no chance of precipitation, and it usually commences in late morning after the morning dew and humidity pass. This is because post-harvest processing (rossing) generally takes place at the collection site outdoors and humidity can damage the quality of the inner bark. Because the inner bark contains polysaccharide mucilage, when it comes in contact with moisture, it begins to gel.

The bark, from trees of a minimum age of 10 years (some harvesters recommend selecting at least 12- to 15-year-old trees) is obtained mainly by pruning or trimming the lower limbs and branches, but can also be obtained from the bole (trunk) and, very rarely, even from the roots in cases where the entire tree is felled. While the inner bark of the root reportedly contains more mucilage than that of the trunk or branches, sustainable wild resource management dictates harvesting only the branches of mature trees. In practice, commercial bark collectors are more likely to select branches from trees that are at least 30 to 50 years old in order to obtain a high enough yield. After pruning, the tree will heal over where harvested and continue to grow, but the trunk and/or branches should never be girdled, because this will kill the tree. Girdling is the stripping away of bark from the trunk or a branch all the way around. In practice, the most sustainable method of harvest from mature healthy trees is to prune off entire lower branches in a way that will not harm the tree. If sawn properly so that rainwater will not drop directly into the exposed cut area, the tree will grow over the cut area within a couple of years. The proper cut, which is made just outside the branch collar and the branch bark ridge, does the least amount of damage to the trees.

Although elm bark should not be gathered from already dead trees, selective collection from dying trees—for example, those affected with Dutch elm disease—is feasible. The fungus that causes Dutch elm disease, *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (syn: *Graphium ulmi* or *Ceratocystis ulmi*), is carried from tree to tree by the European elm bark beetle (*Scolytus multistriatus*), which arrived in North America on a ship carrying logs from Europe around 1930, and to a much lesser extent by the native elm bark beetle (*Hylurgopinus rufipes*). Elms appear to succumb to the disease at about 10 years of age and then die off in a two-year period. Once a tree is diagnosed with Dutch elm disease, healthy bark can still be harvested for about two years, up until the tree is near death. To monitor how long harvesting can continue, one can use a drawknife to take samples. Once the inner bark is showing increasingly noticeable black streaking, and as more and more limbs die and debarking is evident, the tree is near death. The near-dead tree can be dropped, and the remaining healthy bark can be stripped from the entire tree. Discolored (black-streaked) inner bark should be separated out and discarded. Pruning lower limbs from healthy trees and/or selectively harvesting entire older trees that are near death can both be acceptable methods as part of a sustainable resource management plan for a specific area of forest under organic supervision.

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Following organic production system rules (e.g., for harvest site selection, absence of prohibited substances, testing of soil and water, disallowed inputs, periodic residue testing, documentation control, independent inspection) will more likely result in botanical raw material that meets the general requirements for pesticide residue and heavy metals limits, among other potential contaminants. Certified organic production of wild elm bark requires producers to promote ecological balance and conserve biodiversity. Wild collected elm bark that is to be certified organic must be harvested from a designated area that has had no prohibited substance applied to it for a period of 3 years immediately preceding the harvest, and must be harvested in a manner that ensures that such harvesting or gathering will not be destructive to the environment and will sustain the growth and production of the wild crop. Wild crop producers must comply with the same organic system plan requirements and conditions, as applicable to their operation, as their counterparts who produce cultivated crops. The producer of organic wild harvested elm bark must initiate practices to support biodiversity and avoid, to the extent practicable, any activities that would diminish it. Production practices must maintain or improve the natural resources of the operation, including soil, water, wetlands, woodlands, and wildlife. This is accomplished, in part, by developing and executing a resource management plan that requires wild harvest from stable populations, minimizing disruption of priority species/sensitive habitats, avoiding erosion, allowing reestablishment, and monitoring wild crop sustainability.

Cultivation Practices—Even though the commercial supply is harvested from wild populations, slippery elm trees can be propagated by cuttings or by seed. For propagation by seed, the ripe seeds are collected from April to June from healthy and successful (dominant) trees from an area similar to the proposed planting site. A ripeness indicator is when the samaras (fruits) are green. It is best to collect seed from trees within 160 km north or south of the planting site, as potential for success is optimal within this range from the parents. Twenty-five seeds per square foot can be scattered, 0.6 cm deep. Slippery elm may be sown as in its normal cycle in the spring in a raised peat moss soil and sand bed. The seedbeds may need a wire top to protect young seedlings. Germination rate is 10% to 25%, with light germination in summer and increased germination the following spring. The young trees can be transplanted into tree tubes within the first month of germination and field planted after one or two years, depending on the size of the tree tube. The tree saplings must be watered during times of drought and routinely checked for insect predation and indications of fertilization needs.

Optimal Times for Harvest—Harvest should preferably occur in the spring (March to May), but can also take place in the autumn. In the spring, bark is harvested from mature trees (minimum 10 years) when the sap begins to rise.

POST-HARVEST HANDLING

Optimal Handling and Processing Practices—To produce pharmacopeial quality elm inner bark, the outer corky layer of bark must be removed, exposing the inner bark. If post-harvest processing occurs at the wild collection site, the pruned limbs and branches should be placed onto clean tarps and not directly on the ground. The very small branches with leaves are stripped off the pruned limbs by hand and discarded. To optimize conformance to standards for composition, identity, purity, and quality (e.g., NMT 2% of adhering outer bark, NMT 2% foreign organic matter, NMT 10% total ash, and NMT 0.65% acid-insoluble ash), a clean bark rosser (hand tool with handle and knife blade) should be used to shave off the outer bark. The rough, scaly matter on the surface of the bark is called *ross*, and to ross bark is to scrape or shave the outer bark from the limb. An experienced rosser can visually discern that at least 98% of the outer bark has been shaved off. The inner bark is white in color (in the spring; reddish later in the season) in obvious visible contrast to the brown outer bark layer. After most of the outer bark is rossed off, greater care must be exercised to very carefully slice off the remaining thin layer of outer bark so as not to waste any of the inner bark in the process. After removal of the outer bark, the inner bark can then be removed in strips, squares, or chips. An incision is made with a clean knife down the center of the limb. Then a clean crowbar is slipped underneath the incision in order to lift and peel the inner bark off from the cambium. The strips of inner bark are stacked on a clean tarp and later bundled for transport to the drying facility.

Drying—*Elm USP* requires a loss on drying limit of NMT 12%. So long as rain is not expected, fresh elm inner bark can be sun-cured within a temperature range of 32° to 60°. Drying can also be carried out in a warm room with airflow or in a greenhouse. Greenhouse drying takes about 3 to 4 days. Drying indoors can take 5 to 7 days, depending on the heat source. Drying at commercial scale, however, is done typically in enclosed drying chambers, in which time and temperature can be better controlled. The strips of elm bark are placed onto a clean screen floor and dried over about 2 days' time at about 50° with fan-forced heat through the floor. Because of additional phytosanitary requirements for export of tree barks to Europe, higher heat exposure is necessary, usually at least 65° but up to 93° for up to two days. Post-drying, the strips of inner bark can be cut or sawn into pieces of equal length and bound into bundles with wire. The bundles usually consist of flat, oblong pieces, about 30 cm in length and from 10 to 15 cm in width. The bark strips can be stored this way until further processing (e.g., cutting or powdering) is scheduled.

Storage—To maintain pharmacopeial purity and quality (e.g., to prevent accumulation of excess moisture), dried elm inner bark should be preserved in well-closed containers, and stored in a cool, dry place.

ADULTERANTS AND CONTAMINANTS

Common contaminants that could cause a material not to conform with the identification tests in the *Elm* monograph in *USP* would include other plant parts: for example, greater than 2% outer bark, which lacks mucilage. Insufficient shaving or rossing of outer bark could cause the material to exceed the monograph limit of NMT 2% of adhering outer bark. Other possible contaminants would include visible discolored inner bark, although no maximum limit has been established (for example, inner bark with visible black streaking obtained from a diseased tree). Powdered bark can also be adulterated with cornmeal, rice flour, starch, or other starchy substances. Consequences of contamination with outer bark or adulteration with flour or starch are lower mucilage content, lower swelling index value, and correspondingly less of a therapeutic demulcent effect that is mucilage-dependent. Excess outer bark could also cause the material to fail the quantitative standard of NMT 10% total ash. Methods to determine the presence of adulterants include microscopic examination in order to determine the presence of excess outer bark or any other adulterant and the concentration of mucilage cells. The *Elm* mucilage test (*Identification A*) as

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well as a modified swelling volume test (based on the test in the *USP* monograph *Psyllium Husk*) may also be useful to investigate if adulteration is suspected.

⟨2040⟩ DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

INTRODUCTION

This general chapter is provided to determine compliance with the disintegration and dissolution standards for dietary supplements where stated in the individual monographs.

For the purposes of this chapter, dietary supplement dosage forms have been divided into three categories: *Vitamin–Mineral Dosage Forms*, *Botanical Dosage Forms*, and *Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms*. *Vitamin–Mineral Dosage Forms* include articles prepared with vitamins, minerals, or combinations of these dietary ingredients, as described in *Table 1*. *Botanical Dosage Forms* comprise formulations containing ingredients of botanical origin, including plant materials and extracts. *Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms* encompass dietary supplements formulated with lawfully recognized dietary ingredients that are different from those pertaining to the two foregoing categories (e.g., amino acids, chondroitin, and glucosamine).

Where a dietary supplement represents a combination of the categories mentioned above, and there is a difference between the requirements for the individual categories, the more stringent requirement applies. [NOTE—"More stringent requirement" means stricter acceptance criteria and/or milder operational conditions.]

Disintegration and dissolution tests as described in this chapter are quality-control tools to assess performance characteristics of dietary supplement finished dosage forms. These performance standards are intended to detect problems that may arise due to use or misuse, or changes in coatings, lubricants, disintegrants, and other components. These performance tests are also intended to detect manufacturing process issues, such as overcompression and overdrying, that would affect the release characteristics of the final dosage forms. These tests are not intended to be used as a demonstration or as a surrogate for in vivo absorption, bioavailability, or effectiveness, unless an in vitro–in vivo correlation (IVIVC) has been established.

DISINTEGRATION

This test is provided to determine whether dietary supplement capsules or tablets disintegrate within the prescribed time when placed in a liquid medium at the experimental conditions presented below. Compliance with the limits on *Disintegration* stated in the individual monographs for dietary supplements is required, except where the label states that the products are intended for use as troches, are to be chewed, or are designed as extended-release dosage forms. Dietary supplements claiming to be extended-release dosage forms must comply with standards other than disintegration to verify that the release of the dietary ingredients from the dosage form is for a defined period of time. Dietary supplements claiming to be extended-release dosage forms must not be labeled as in compliance with USP unless a *USP* monograph exists for such product. Determine the type of dosage form under test from the labeling and from observation, and apply the appropriate procedure to 6 or more units.

For purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk (if used) is a soft mass having no palpably firm core.

• APPARATUS

Apparatus A: Use the *Apparatus* described in *Disintegration* ⟨701⟩, *Apparatus* for capsules or tablets that are NMT 18 mm long. For larger capsules or tablets, use *Apparatus B*.

Apparatus B: The apparatus consists of a basket-rack assembly, a 1000-mL low-form beaker for the immersion fluid, a thermostatic arrangement for heating the fluid between 35° and 39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles/min through a distance of 53–57 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke, the wire mesh remains at least 15 mm below the surface of the fluid and descends to NLT 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

Basket-rack assembly: The basket-rack assembly (see *Figure 1*) consists of three open-ended transparent tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 32.0–34.6 mm and a wall 2.0–3.0 mm in thickness; the tubes are held in a vertical position by two plastic plates, each 97 ± 2 mm in diameter and 7.5–10.5 mm in thickness, with three holes, 36.0–40.6 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the undersurface of the lower plate is 10-mesh No. 23 (0.025-inch) W- and M-gauge woven stainless-steel wire cloth having a plain square weave. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plastic plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device, using a point on its axis. The design of the basket-rack assembly may be varied somewhat, provided that the specifications for the glass tubes and the screen mesh size are maintained.

Beaker: Low form, 1000 mL; the difference between the diameter of the plastic plates, which hold the tubes in a vertical position, and the inside diameter of the beaker should be NMT 6 mm.[1]

Disks: Each tube is provided with a perforated cylindrical disk 15.3 ± 0.15 mm in thickness and 31.4 ± 0.13 mm in diameter. The disk is made of a suitable, transparent plastic material having a specific gravity of between 1.18 and

[1] 1000-mL low-form beakers, designed in compliance with the current ASTM E 960 Type I or Type II or ISO 3819 specifications, meet the size requirements.

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1.20. Seven holes 3.15 ± 0.1 mm in diameter extend between the ends of the cylinder, one of the holes being in the center and the other six parallel to it and spaced equally tangent to a circle with a radius of 4.2 mm from the center of the disk. All surfaces of the disk are smooth.[2]

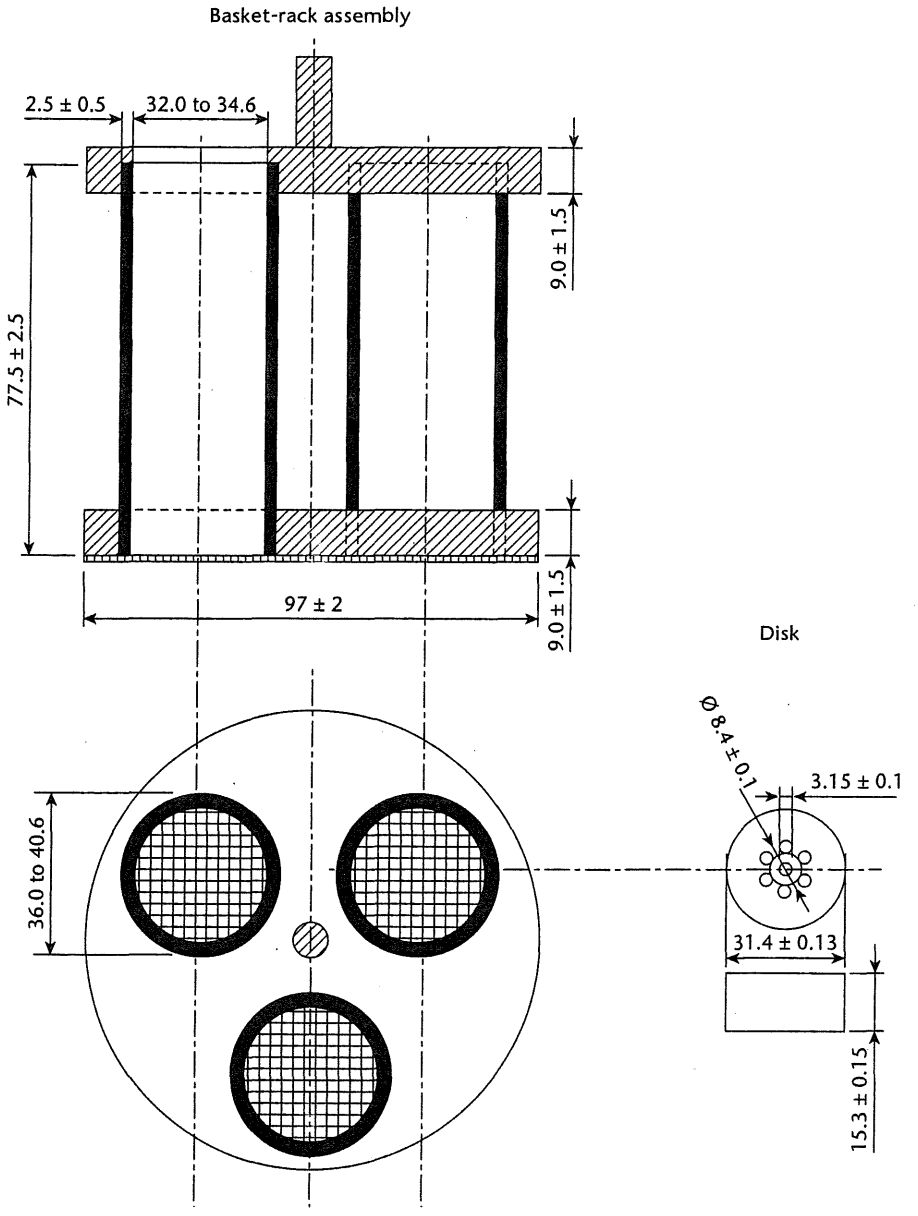

Figure 1. Basket-rack assembly, *Disintegration, Apparatus B* (dimensions in mm).

• **PROCEDURE:** Test 6 dosage units as described below for each type of dosage form. [NOTE—Two basket arrangements for a total of six tubes are necessary for *Apparatus B.*] If 1 or 2 dosage units fail to disintegrate completely, repeat the test on 12 additional dosage units.

Uncoated tablets: Place 1 tablet in each of the tubes of the basket and, if prescribed, add a disk to each tube. Operate the apparatus, using water or the specified medium as the immersion fluid, maintained at 37 ± 2°. At the end of 30 min, lift the basket from the fluid and observe the tablets.

Plain-coated tablets: Place 1 tablet in each of the tubes of the basket and, if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 min. Then, if prescribed, add a disk to each tube and

[2] The use of automatic detection using modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimensions given in this chapter.

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operate the apparatus, using water or the specified medium as the immersion fluid, maintained at 37 ± 2°. At the end of 30 min, lift the basket from the fluid and observe the tablets.

Delayed-release (enteric-coated) tablets: Omit the use of a disk. Place 1 tablet in each of the six tubes of the basket, and if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 min. Then operate the apparatus using simulated gastric fluid TS, maintained at 37 ± 2°, as the immersion fluid. After 1 h of operation in simulated gastric fluid TS, lift the basket from the fluid and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Operate the apparatus using simulated intestinal fluid TS, maintained at 37 ± 2°, as the immersion fluid for the time specified in the monograph. Lift the basket from the fluid and observe the tablets.

Delayed-release (enteric-coated) soft-shell capsules: Place 1 softgel capsule in each of the six tubes of the basket. Omit the use of a disk. Operate the apparatus using simulated gastric fluid TS, maintained at 37 ± 2°, as the immersion fluid. After 1 h of operation in simulated gastric fluid TS, lift the basket from the fluid and observe the softgels: the softgels show no evidence of disintegration or rupture that would permit the escape of the contents. Operate the apparatus with disks using simulated intestinal fluid TS, maintained at 37 ± 2°, as the immersion fluid for NMT 60 min. Lift the basket from the fluid and observe the capsules.

Hard-shell capsules: Apply the test for *Uncoated tablets* using, as the immersion fluid, maintained at 37 ± 2°, a 0.05 M acetate buffer prepared by mixing 2.99 g of sodium acetate trihydrate and 1.66 mL of glacial acetic acid with water to obtain a 1000-mL solution with a pH of 4.50 ± 0.05. Attach a removable wire cloth, as described in *Basket-rack assembly*, to the surface of the upper plate of the basket-rack assembly. At the end of 30 min, lift the basket from the fluid and observe the capsules.

Soft-shell capsules: Proceed as directed in the *Rupture Test for Soft-Shell Capsules*.

• **USE OF DISKS**

Vitamin–mineral dosage forms: Add a disk to each tube unless otherwise specified in the *Procedure* above or in the individual monograph.

Botanical dosage forms: Omit the use of disks unless otherwise specified in the *Procedure* above or in the individual monograph.

Dietary supplements other than vitamin–mineral and botanical dosage forms: Omit the use of disks unless otherwise specified above or in the individual monograph.

• **TOLERANCES:** All of the 6 dosage units initially tested or NLT 16 of a total of 18 dosage units tested disintegrate completely.

RUPTURE TEST FOR SOFT-SHELL CAPSULES

Medium: Water; 500 mL

Apparatus: Use *Apparatus 2* as described in *Dissolution* ⟨711⟩, *Apparatus*, operating at 50 rpm.

Time: 15 min

• **PROCEDURE:** Place 1 capsule in each vessel, and allow the capsule to sink to the bottom of the vessel before starting rotation of the blade. Use sinkers if the capsules float. Observe the capsules throughout the test and at the end of the test. The capsule shell is considered ruptured if breached, exposing or allowing the fill contents to escape.

• **TOLERANCES:** The requirements are met if all of the capsules tested rupture in NMT 15 min. If 1 or 2 of the capsules rupture in >15 min but NMT 30 min, repeat the test on 12 additional capsules: NMT 2 of the total of 18 capsules tested rupture in >15 min but NMT 30 min. For soft gelatin capsules that do not conform to the above rupture test acceptance criteria, repeat the test with the addition of papain to the *Medium* in the amount that results in an activity of NMT 550,000 units/L of *Medium* or with the addition of bromelain in the amount that results in an activity of NMT 30 gelatin-digesting units (GDU)/L of *Medium*. [NOTE—Determine papain activity using the *Assay* in the monograph for *Papain* and bromelain activity using the procedure in bromelain, in the *Reagent Specifications* section.]

DISSOLUTION

This test is provided to determine compliance with the *Dissolution* requirements where stated in the individual monographs for dietary supplements. The operative assumption inherent in this test is that if the index vitamin or mineral or marker compound(s) for a botanical is dissolved within the time frame and under conditions specified, the dosage form does not suffer from formulation- or manufacturing-related problems affecting the adequate release of the active ingredients.

• **FOR DOSAGE FORMS CONTAINING OR COATED WITH GELATIN**

For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the dissolution specification because of the presence of cross-linking, the dissolution procedure should be repeated with the addition of enzymes to the medium, as described below.

Dissolution medium with pH ≤4.0

Enzyme: Pepsin, activity determined by the procedure in pepsin, in the *Reagent Specifications* section

Amount: A quantity of pepsin that results in an activity of NMT 750,000 units/L of dissolution medium

Dissolution medium with pH >4.0 and <6.8

Enzyme: Papain, activity determined by the *Assay* in the monograph for *Papain*; or bromelain, activity determined by the procedure in bromelain, in the *Reagent Specifications* section

Amount: A quantity of papain that results in an activity of NMT 550,000 units/L of dissolution medium, or a quantity of bromelain that results in an activity of NMT 30 GDU/L of dissolution medium

Dissolution medium with pH ≥6.8

Enzyme: Pancreatin, protease activity determined by the procedure in *Assay for protease activity* (*Casein digestive powder*) in the monograph for *Pancreatin*

Amount: A quantity of pancreatin that results in a protease activity of NMT 2,000 units/L of dissolution medium

Dissolution medium containing surfactants or other components known to denature the enzyme: If the dissolution medium contains surfactants or other components known to denature the enzyme to be used, a pretreatment step should be applied. The pretreatment step is performed under the same dissolution conditions (apparatus, rotation, and flow rate), except to use a medium with the corresponding amount of enzyme as directed in the preceding section and without the surfactant or component known to denature the enzyme. To achieve the final specified volume of medium,

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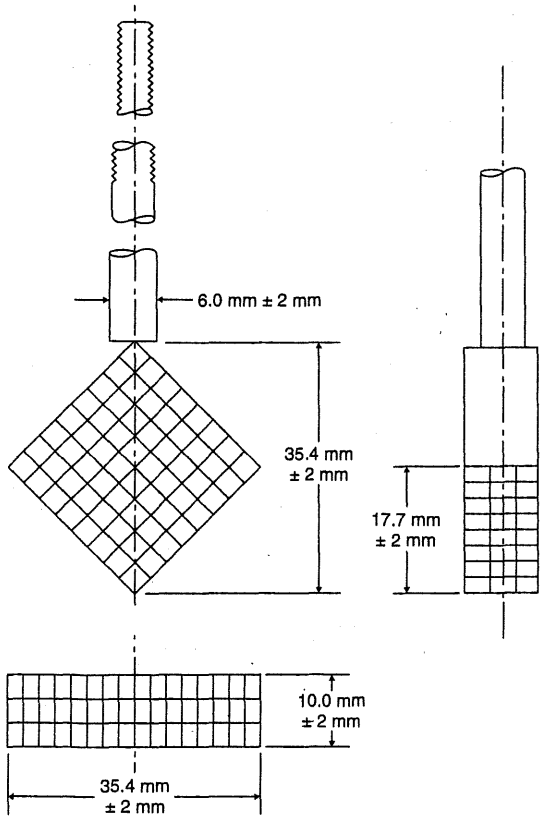
the pretreatment step may be conducted with a smaller volume of medium without the surfactant or component in such a manner that the final specified volume is achieved after the addition of the surfactant or component at the end of the pretreatment step. Perform the pretreatment step until capsule rupture, but for NMT one-half of the total dissolution time specified in the procedure. The pretreatment time is included in the total dissolution time specified in the procedure.

• **APPARATUS:** See ⟨711⟩ for a description of the apparatus used, apparatus suitability test, and other related information. Where the procedure specifies the use of a stationary basket, use the quadrangular basket of stainless steel wire gauze as shown in *Figure 2a* and *Figure 2b*.

The capsule is placed in a basket, soldered in one of its upper, narrow sides to the end of a steel rod (see *Figure 2a*). The capsule cover is placed in the horizontal diagonal of the basket. The rod assembly is inserted vertically through the cover of the dissolution vessel, and fixed by means of two teflon nuts, 3.2 cm from the center of the vessel, or by any other appropriate means. The lower edge of the bottom of the basket is adjusted to about 1 cm above the top of the paddle blade (see *Figure 2b*).



NOTES

1. Rod and Basket with a Capsule cover placed in the horizontal diagonal of the basket
2. Basket and capsule cover material; stainless steel
3. Basket gauze wire size: 8 mesh

Figure 2a. Stationary Basket

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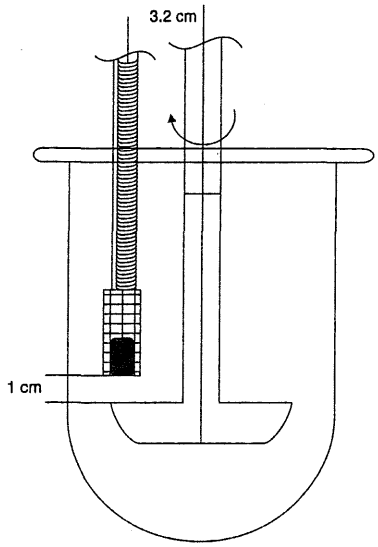

Figure 2b. Stationary Basket Configuration Diagram

Of the types of apparatus described in ⟨711⟩, use the one specified in the individual monograph.

• **VITAMIN–MINERAL DOSAGE FORMS**

All dietary supplement capsules, tablets, or chewable gels containing folic acid are subject to the dissolution test and criteria for folic acid described in this chapter. This test is required because of the importance of the relationship between folate deficiency and the risk of neural tube defects. Dietary supplement capsules, tablets, or chewable gels containing water-soluble vitamins, minerals, or their combination are subject to the dissolution test and criteria for index vitamins, index minerals, or both, described in this chapter. Dietary supplement tablets, chewable gels, and hard-shell capsules with solid content dosage forms containing vitamin A are subject to the dissolution test and criteria for vitamin A described in this chapter. Dissolution standards were not established and therefore are not applicable to vitamin A in dietary supplement soft-shell capsules filled with liquids. *Table 1* summarizes the dissolution requirements for the assigned USP classes of dietary supplements. Vitamin–mineral combinations that do not belong to any of the USP classes listed in *Table 1* are subject to the *Dissolution* test and criteria specified in the individual monographs.

Table 1. Dietary Supplements—Vitamin–Mineral Dosage Forms

USP Class	Ingredients	Dissolution Requirements for Tablets, Chewable Gels, and Hard-Shell Capsules with Solid Contents	Dissolution Requirements for Soft-Shell Capsules Filled with Liquids
I	Oil-soluble vitamins	Vitamin A (if present)	Not applicable
II	Water-soluble vitamins	One index water-soluble vitamin and folic acid (if present)	One index water-soluble vitamin and folic acid (if present)
III	Water-soluble vitamins with minerals	One index water-soluble vitamin, one index element, and folic acid (if present)	One index water-soluble vitamin, one index element, and folic acid (if present)
IV	Oil- and water-soluble vitamins	Vitamin A (if present), one index water-soluble vitamin, and folic acid (if present)	One index water-soluble vitamin and folic acid (if present)
V	Oil- and water-soluble vitamins with minerals	Vitamin A (if present), one index water-soluble vitamin, one index element, and folic acid (if present)	One index water-soluble vitamin, one index element, and folic acid (if present)
VI	Minerals	One index element	One index element
VII	Oil-soluble vitamins with minerals	Vitamin A (if present) and one index element	One index element

Selection of index water-soluble vitamins and index elements: Compliance with the dissolution requirements for dietary supplements representing combinations of water-soluble vitamins and combinations of oil- and water-soluble vitamins is determined by measuring the dissolution of a single index vitamin from the water-soluble vitamins present. Riboflavin is the index vitamin when present in the formulation. For formulations that do not contain riboflavin, pyridoxine is the index vitamin. If neither riboflavin nor pyridoxine is present in the formulation, the index vitamin is niacinamide (or niacin), and in the absence of niacinamide (or niacin), the index vitamin is thiamine. If none of these four water-soluble vitamins are present in the formulation, the index vitamin is ascorbic acid.

Compliance with the dissolution requirements for dietary supplements representing combinations of minerals is determined by measuring the dissolution of only one index element. Iron is the index element when present in the formulation. For formulations that do not contain iron, the index element is calcium. If neither iron nor calcium is present, the index element is zinc. In the absence of all three of these elements, magnesium is the index element.

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Compliance with the dissolution requirements for dietary supplements representing combinations of water-soluble vitamins and minerals and combinations of oil- and water-soluble vitamins and minerals is determined by measuring the dissolution of one index water-soluble vitamin and one index element, designated according to the respective hierarchies described above.

Dissolution conditions for vitamin A: [NOTE—Perform this test under light conditions that minimize photodegradation.]
Medium: 1% (w/v) sodium ascorbate and 1% (w/v) octoxynol 9 in 0.05 M phosphate buffer, pH 6.8; 900 mL
Apparatus 2: 75 rpm
Time: 45 min

Dissolution conditions for folic acid: [NOTE—Perform this test under light conditions that minimize photodegradation.]
Test 1
Medium: Water; 900 mL
Apparatus 1: 100 rpm, for capsules
Apparatus 2: 75 rpm, for tablets
Time: 1 h
If the units tested do not meet the requirements for dissolution in water, use the following conditions:
Buffer: Mix 95 mL of 0.1 M citric acid monohydrate and 405 mL of 0.1 M sodium citrate dihydrate, dilute with water to 1000 mL, mix, and adjust to a pH of 6.0 by using either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution.
Medium: Buffer; 900 mL
Apparatus 1: 100 rpm, for capsules
Apparatus 2: 75 rpm, for tablets
Time: 1 h
Test 2 (for lipid-filled soft-shell capsules): Proceed as directed for *Test 2* under *Dissolution conditions for index water-soluble vitamins and index minerals*. If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 2*.
Test 3 (for lipid-filled soft-shell capsules): Proceed as directed for *Test 3* under *Dissolution conditions for index water-soluble vitamins and index minerals*. If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 3*.
[NOTE—Compliance with the dissolution requirements for folic acid does not exempt the article from compliance with the dissolution requirements of the pertinent index vitamin or the corresponding index mineral.]

Dissolution conditions for index water-soluble vitamins and index minerals
Test 1
Medium: 0.1 N hydrochloric acid; 900 mL
Apparatus 1: 100 rpm, for capsules
Apparatus 2: 75 rpm, for tablets
Time: 1 h
For formulations containing 25 mg or more of the index vitamin, riboflavin, use the following conditions:
Medium: 0.1 N hydrochloric acid; 1800 mL
Apparatus 1: 100 rpm, for capsules
Apparatus 2: 75 rpm, for tablets
Time: 1 h
Test 2 (for lipid-filled soft-shell capsules): If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 2*.
Medium: 0.25% (w/v) octoxynol 9, 0.02% (w/v) ascorbic acid, and 0.04% (w/v) simethicone in simulated gastric fluid TS; 250 mL
Apparatus 3: 15 dpm
Screen (top and bottom): 20-mesh
Time: 1 h
Test 3 (for lipid-filled soft-shell capsules): If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 3*.
Medium: 0.25% (w/v) octoxynol 9 and 0.02% (w/v) ascorbic acid in simulated gastric fluid TS; 500 mL
Apparatus 2: 125 rpm; dosage unit placed in stationary basket (*Figure 2a* and *Figure 2b*)
Time: 1 h
[NOTE—Compliance with dissolution requirements for the pertinent index vitamin or index mineral does not exempt the article from compliance with the dissolution requirements for folic acid, if present.]

Procedures: In the following procedures, combine equal volumes of the filtered solutions of the six individual specimens withdrawn, and use the pooled sample as the test specimen. Determine the average amount of vitamin A, folic acid, or the index vitamin or element dissolved in the pooled sample. Make any necessary modifications, including concentration of the analyte in the volume of *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and dilution, if necessary, of the *Sample solution*.
Vitamin A: Determine the percentage of retinyl acetate or retinyl palmitate dissolved by using the following procedure.
Standard solution: Dissolve a suitable amount of USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl alcohol, and dilute with *Medium* to obtain a concentration similar to that expected in the *Sample solution*. [NOTE—The amount of isopropyl alcohol should be 5%–10%.]
Sample solution: Withdraw a portion of the solution under test, pass through a suitable filter of 0.45-µm pore size, and use the pooled sample as the test specimen.
Solution A: Methanol and water (90:10)
Solution B: Methanol and isopropyl alcohol (55:45)
Mobile phase: See *Table 2*.

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Table 2

Time (min)	Solution A (%)	Solution B (%)
0	100	0
8	0	100
13	0	100
13.1	100	0
15	100	0

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 10-cm; 3-µm packing L1
Flow rate: 1.0 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 1.5 for retinyl acetate; NMT 2.0 for retinyl palmitate
Relative standard deviation: NMT 2.0%
Analysis
Samples: Appropriate *Standard solution* and *Sample solution*

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*
C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)
V = volume of *Medium*, 900 mL
L = label claim of vitamin A, as retinol (µg/tablet)

Folic acid: Determine the amount of folic acid ($C_{19}H_{19}N_7O_6$) dissolved by using the procedure set forth in the assay for folic acid in the individual monograph. Make any necessary modifications.
Niacin or niacinamide, pyridoxine, riboflavin, and **thiamine:** Determine the amount of the designated index vitamin dissolved by using the procedure set forth in the assay for niacin or niacinamide, pyridoxine hydrochloride, riboflavin, and thiamine in the individual monographs. Make any necessary modifications.
Ascorbic acid: Determine the amount of ascorbic acid ($C_6H_8O_6$) dissolved by using the procedure set forth in the assay for ascorbic acid in the individual monograph. Make any necessary modifications.
Iron, calcium, magnesium, and **zinc:** Determine the amount of the designated index element dissolved by using the procedure set forth in the appropriate assay in the individual monographs. Make any necessary modifications.
Tolerances: The requirements are met if NLT 75% of the labeled content of vitamin A, NLT 75% of the labeled content of folic acid, and NLT 75% of the labeled content of the index vitamin or the index element from the units tested is dissolved.

• **Botanical Dosage Forms**
Compliance with dissolution requirements necessitates the testing of 6 dosage units individually, or testing 2 or more dosage units in each of the six vessels of the dissolution apparatus, and measuring the dissolution of one or more index/marker compound(s) or the extract specified in the individual monograph.
Procedures: Combine equal volumes of the filtered solutions of the six or more individual specimens withdrawn, and use the pooled sample as the *Sample solution*. Determine the average amount of index or marker compound(s) or the extract dissolved in the pooled sample by the procedure specified in the individual monograph. Make any necessary modifications, including concentration of the analyte in the volume of the *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and dilution, if necessary, of the *Sample solution*.
Tolerances: Unless otherwise specified in the individual monograph, the requirements are met if NLT 75% of the labeled content of the index or marker compound(s) or the extract from the units tested is dissolved in 1 h.

• **Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms**
Unless otherwise stated in the individual monographs for dietary supplement dosage forms in this category, compliance requires the testing of 6 individual units, measuring the dissolution of the dietary ingredient as the average of the 6 units tested.
Procedures: Combine equal volumes of the filtered solutions of the six specimens withdrawn, and use the pooled sample as the *Sample solution*. Determine the average amount of the dietary ingredient dissolved in the pooled sample by the procedure specified in the individual monograph. Make any necessary modifications, including concentration of the analyte in the volume of the *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and for dilution, if necessary, of the *Sample solution*.

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Tolerances: Because of the diversity of chemical characteristics and solubilities of dietary ingredients pertaining to this category, general tolerances cannot be established. See individual monographs for *Tolerances*.

⟨2091⟩ WEIGHT VARIATION OF DIETARY SUPPLEMENTS

Change to read:

INTRODUCTION

The following tests provide limits for the permissible variations in the weights of individual tablets, capsules, ▲or chewable gels▲ (USP 1-Dec-2019) expressed in terms of the allowable deviation from the average weight of a sample. Separate procedures and limits are described herein for capsules, uncoated tablets, coated tablets, ▲and chewable gels▲ (USP 1-Dec-2019) that are intended for use as dietary supplements.

CAPSULES

Capsules meet the requirements of the following test with respect to variation in weight of contents.

Hard Shell Capsules

Weigh 20 intact capsules individually, and determine the average weight. The requirements are met if each of the individual weights is within the limits of 90% and 110% of the average weight.

If not all of the capsules fall within the aforementioned limits, weigh the 20 capsules individually, taking care to preserve the identity of each capsule, and remove the contents of each capsule with the aid of a small brush or pledget of cotton. Weigh the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight. Determine the average net content from the sum of the individual net weights. Then determine the difference between each individual net content and the average net content: the requirements are met if (a) NMT 2 of the differences are greater than 10% of the average net content and (b) in no case is the difference greater than 25%.

If more than 2 but NMT 6 capsules deviate from the average between 10% and 25%, determine the net contents of an additional 40 capsules, and determine the average content of the entire 60 capsules. Determine the 60 deviations from the new average: the requirements are met if (a) NMT 6 of the 60 capsules have differences that are greater than 10% of the average net content and (b) in no case does the difference exceed 25%.

Soft Shell Capsules

Proceed as directed under *Hard Shell Capsules*, but determine the net weight of the contents of individual capsules as follows. Weigh the intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument, such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. The requirements are as stated under *Hard Shell Capsules*.

TABLETS

Tablets conform to the criteria given in *Table 1*.

Uncoated Tablets and Film-Coated Tablets

Weigh 20 whole tablets individually, and calculate the average weight. The requirements are met if the weights of NMT 2 of the tablets differ from the average weight by more than the percentage listed in *Table 1*, and no tablet differs in weight by more than double that percentage.

Coated Tablets (Other Than Film-Coated Tablets)

Weigh 20 whole tablets individually, and calculate the average weight. If the coated tablets do not conform to the criteria in *Table 1*, place 20 tablets in a beaker of water at 37° and swirl gently for NMT 5 min. Examine the cores for evidence of disintegration and repeat the procedure for a shorter time if disintegration has begun. Dry the cores at 50° for 30 min. Accurately weigh 20 individual tablet cores, and calculate the average weight.

The requirements are met if the weights of NMT 2 of the tablets differ from the average weight by more than the percentage listed in *Table 1* and no tablet differs in weight by more than double that percentage.

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Acceptance Criteria

Table 1. Weight Variation Tolerances for Uncoated Tablets, Film-Coated Tablets, and Coated Tablets (Other Than Film-Coated Tablets)

Average Weight of Tablet (mg)	Difference (%)
130 or less	10
From 130 through 324	7.5
More than 324	5

Add the following:

▲CHEWABLE GELS

Chewable gels meet the requirements of the following test with respect to weight variation. Individually weigh an equal number of units of each color and shape to obtain a total of NLT 20 and NMT 30 individual weights, and calculate the average weight.

The requirements are met if no individual weight deviates from the average weight by more than 7.5%. If more than 1 unit exceeds the specified limit, the test fails.

If 1 unit falls outside of the limits, repeat the procedure with an additional set of NLT 20 and NMT 30 individual chewable gels. The requirements are met if none of the units tested in the second set differ from their average weight by more than 10%.▲ (USP 1-Dec-2019)

⟨2232⟩ ELEMENTAL CONTAMINANTS IN DIETARY SUPPLEMENTS

The objective of this general chapter is to limit the amounts of elemental contaminants in finished dietary supplement dosage forms labeled as conforming to *USP* or *NF* standards. This general chapter is not intended to set limits for dietary ingredients. Those limits are set in the corresponding individual monographs.

The focus of this general chapter is on the four major elements of toxicological concern: arsenic, cadmium, lead, and mercury. The extent of testing can be determined using a risk-based approach that takes into account the likelihood of contamination. Manufacturers should consider the presence of unexpected elemental contaminants to determine compliance.

LIMITS OF ELEMENTAL CONTAMINANTS

The levels of elemental contaminants should be restricted as shown in *Table 1* unless otherwise stated in the individual monograph. Specific monographs may provide different limits for articles that need to be consumed in large quantities.

Table 1

Element	PDE (µg/day)[a]
Arsenic (inorganic)	15
Cadmium	5
Lead	5
Mercury (total)	15
Methylmercury (as Hg)	2

[a] Permitted Daily Exposure (PDE) is derived from the Provisional Tolerable Weekly Intake (PTWI) that is recommended by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) by subtracting the daily exposure (µg/day) to each elemental contaminant from air, food, and drinking water. A body weight of 50 kg and a safety factor are used to calculate the PDE. Other regulations (e.g., Proposition 65 in California) may require different limits; manufacturers are responsible for compliance with applicable local requirements differing from these PDE values.

Arsenic may be measured using a nonspeciation procedure under the assumption that all arsenic contained in the supplement is in the inorganic form. Where the limit is exceeded using a nonspeciation procedure, compliance with the limit for inorganic arsenic shall be demonstrated on the basis of a speciation procedure. Methylmercury determination is not necessary when the content for total mercury is less than the limit for methylmercury.

OPTIONS FOR COMPLIANCE WITH THE LIMITS OF ELEMENTAL CONTAMINANTS

In order for a dietary supplement finished dosage form to comply with the limits for elemental contaminants as described in this chapter, the level of elemental contaminant in the finished dietary supplement should be NMT the PDE. The following three options are available for determining compliance with the limits for elemental contaminants in dietary supplements.

- **Dietary Supplement Analysis Option**

  This option is generally applicable. In this option the finished dietary supplement dosage form is analyzed according to the procedures in the general chapter *Elemental Impurities—Procedures* ⟨233⟩ or the speciation procedures given in this chapter. The results obtained from the analysis of a typical serving size, scaled to a maximum daily intake, are compared to the PDE, as stated in *Table 1*.

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Analysis: Proceed as directed below in this chapter.
Calculate the measured amount of each elemental contaminant, in µg/daily intake, as:

$$\text{Result} = MVSS \times N$$

$MVSS$ = measured amount of each elemental contaminant (µg/serving size)
N = maximum daily intake of the supplement recommended in the labeling (servings/day)

Acceptance criteria: The measured amount/daily intake is NMT the PDE value given in *Table 1*.

• **INDIVIDUAL COMPONENT OPTION**

This option is applicable to finished dietary supplement dosage forms with a maximum daily intake of NMT 10 g of the dietary supplement finished product.

Analysis: Unless otherwise specified in the individual monograph, proceed with the individual ingredient as directed below in this chapter.

Acceptance criteria: The product meets the requirements when each component used to prepare the finished dietary supplement meets the limits given in *Table 2*.

Table 2

Element	Individual Component Limits (µg/g)[a]
Arsenic (inorganic)[b]	1.5
Cadmium	0.5
Lead	0.5
Mercury (total)	1.5
Methylmercury (as Hg)[c]	0.2

[a] The limits for individual components are based on a maximum daily intake of 10 g of a dietary supplement and are intended for use only with *Options for Compliance with the Limits of Elemental Contaminants, Individual Component Option.*
[b] Arsenic may be measured using a nonspeciation procedure under the assumption that all arsenic contained in the supplement is in the inorganic form. Where the limit is exceeded using a nonspeciation procedure, compliance with the limit for inorganic arsenic shall be demonstrated on the basis of a speciation procedure.
[c] Methylmercury determination is not necessary when the content for total mercury is less than the limit for methylmercury.

[NOTE—If all components in a formulation meet the limits given for the *Individual Component Limits*, these components can be used in any proportion. No further calculation is necessary.]

• **SUMMATION OPTION**

This option can be used for finished dietary supplement dosage forms that are consumed in quantities greater than 10 g/day or where the acceptance limit for any contaminant in any component of the dietary supplement exceeds the applicable *Individual Component Limits*.

Analysis: Unless otherwise specified in the individual monograph, proceed with the individual ingredient as directed below in this chapter.

Calculate the amount of each elemental contaminant, in µg/daily intake, present in the finished dietary supplement dosage form:

$$\text{Result} = \Sigma(C_i \times W_i) \times N$$

C_i = elemental contaminant concentration in the individual component (µg/g)
W_i = weight of each individual component per serving of the dietary supplement (g/serving)
N = maximum daily intake of the supplement recommended in the labeling (servings/day)

Acceptance criteria: The calculated amount of each elemental contaminant/daily intake is NMT the PDE value given in *Table 1*.

ANALYTICAL PROCEDURES FOR TOTAL ELEMENTAL CONTAMINANTS

Performance-based methodology for analysis of total elemental contaminants in general chapter *Elemental Impurities—Procedures* ⟨233⟩ is applicable for dietary supplements. The validation necessity will vary depending on the situation. In all three options described in the section *Options for Compliance with the Limits of Elemental Contaminants*, the use of *Validation of Limit Procedures* (see *Elemental Impurities—Procedures* ⟨233⟩) may be appropriate. However, for the *Summation Option*, acceptable levels of validation must be determined on a case-by-case basis. Validation of a procedure using the *Validation of Quantitative Procedures* (see *Elemental Impurities—Procedures* ⟨233⟩) is acceptable for all options under all circumstances and is generally preferred. The determination of the level of validation necessity is at the discretion of the manufacturer and the competent regulatory authority.

• **ANALYTICAL PROCEDURE FOR INORGANIC ARSENIC**

Where the level of total arsenic exceeds the limit recommended in this chapter, speciation may be used to determine the amount of inorganic arsenic present. The following procedure is suggested for determination of inorganic arsenic, but any validated procedure shown to give equivalent or better results can be used.

Apparatus: See *Figure 1*.

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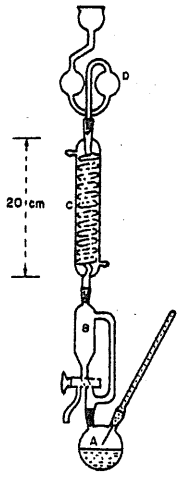

Figure 1. Special apparatus for the determination of inorganic arsenic (A, 250-mL distillation flask; B, receiver chamber, approximately 50-mL capacity; C, reflux condenser; D, splash head or security funnel).

Reagents

Distillation-reducing solution: 30.0 g of potassium iodide in 100 mL of water. [NOTE—Prepare fresh on the day of use.]

Control: 6.0 µg of arsenic (As) (6.0 mL of *Standard Arsenic Solution*). [NOTE—Use this amount rather than the 3.0 mL specified for *Standard Preparation* in the general chapter *Arsenic* ⟨211⟩, *Method I*.]

Sample solution: Transfer a 2.00-g sample that has previously been ground to pass through a 60-mesh screen to a distillation flask (A). To the flask add 35 mL of hydrochloric acid 6.6 N and 15 mL of *Distillation-reducing solution*, let stand for 5 min to ensure reduction of arsenic [As(V)], connect the flask to the receiver chamber (B), complete the assembly of the apparatus, and begin circulating tap water through the condenser (C). Half-fill the lower two bulbs of the splash head (D) with water. Maneuver the stopcock to cause the contents of the receiver chamber to drain into the distillation flask, heat the flask until the temperature above the solution reaches 100°–110°, and continue refluxing at this temperature for 15 min. Close the stopcock, continue heating at 108°–110°, and collect 30–33 mL of distillate in the receiver chamber. Remove the heating source, and allow the temperature to drop to about 80°.

Close the stopcock, and add a second 35-mL portion of 6.6 N hydrochloride (HCl) and 15 mL of the *Distillation-reducing solution* through the thermometer opening to the distillation flask. Replace the thermometer, increase the temperature to 100°–110°, and collect a second 30- to 33-mL portion of distillate in the receiver chamber. Drain the second distillate into the beaker containing the first portion, and cool the combined distillate to room temperature. Remove the splash head, and wash its contents into the beaker. Also, wash down the inside of the condenser and receiver chamber with water, collecting the washings into the beaker. Transfer to a 100-mL volumetric flask, and complete with water to volume.

Analysis: Determine the arsenic content by the ICP-MS procedure in *Elemental Impurities—Procedures* ⟨233⟩. Alternatively, add 2 mL of potassium iodide TS and 0.5 mL of stronger acid stannous chloride TS to the *Sample solution* contained in the Erlenmeyer flask, and proceed as directed in *Arsenic* ⟨211⟩, *Method I*, *Procedure*, beginning with "Allow to stand at room temperature for 30 minutes."

• **ANALYTICAL PROCEDURE FOR METHYLMERCURY**

Where methylmercury determination is required, the following procedure is suggested. However, any validated procedure shown to give equivalent or better results can be used.

Procedure 1

This procedure uses an aqueous extraction of the mercury species with an L-cysteine solution, HPLC separation of the derivatized mercury species with a mobile phase also containing L-cysteine, and ICP-MS detection.

Cysteine solution: 1% L-cysteine hydrochloride monohydrate in water

Mobile phase: 0.1% L-cysteine hydrochloride monohydrate and 0.1% L-cysteine free base in water

Standard stock solution: 1 µg/mL of mercury (Hg) as methylmercury chloride, and 1 µg of mercury (Hg) as mercury chloride in 5% hydrochloric acid containing 0.2 mg/mL of L-cysteine hydrochloride monohydrate

Standard solution: 2 ng/mL of mercury as methylmercury from *Standard stock solution* in *Cysteine solution*

Sample solutions

For supplements in tablet form: Weigh, and finely powder a counted number of tablets. Transfer an accurately weighed portion of the powder equivalent to 0.5 times the daily recommended intake to a tared vial. Add 50.0 mL of *Cysteine solution* accurately weighed, cap the vial, and shake vigorously. Place the vial in a water bath at 60° for 60 min. Shake the vial again, and return to the water bath for another period of 60 min. Shake the vial again, and allow to cool at room temperature for about 20 min. Filter through a 0.45-µm polyethylene membrane. [NOTE—Prepare fresh on the day of use.]

For supplements in capsule form: Weigh accurately NLT 20 capsules, and determine the average weight. Place a number of capsules equivalent to about 5 times the daily recommended intake in a blender, add 500.0 mL of *Cysteine solution* and blend to obtain a homogenous analytical suspension. Transfer 50.0 mL of this analytical suspension to a vial, cap the vial, and shake vigorously. Place the vial in a water bath at 60° for 60 min. Shake the vial again, and

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return to the water bath for another period of 60 min. Shake the vial again, and allow to cool at room temperature for about 20 min. Filter through a 0.45-µm polyethylene membrane.

[NOTE—Prepare fresh on the day of use.]

For supplements in liquid form: Weigh accurately an amount of the liquid equivalent to 0.5 times the daily recommended intake into a vial, and add 50.0 mL of *Cysteine solution*. Cap the vial, and shake vigorously. Place the vial in a water bath at 60° for 60 min. Shake the vial again, and return to the water bath for another period of 60 min. Shake the vial again, and allow to cool at room temperature for about 20 min. Filter through a 0.45-µm polyethylene membrane.

[NOTE—Prepare fresh on the day of use.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: ICP-MS at a mass-to-charge ratio of 202

Column: 4.6-mm × 15-cm; 4-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 3 between the peak representing mercury (Hg^{2+}) species at a relative retention time of about 0.56 and the peak representing methylmercury ($MeHg^{+}$) species at a relative retention time of 1.0

Relative standard deviation: NMT 10.0%, *Standard solution*

Analysis

Samples: *Standard solution* and the appropriate *Sample solution*

Determine the peak area of the peak representative of methylmercury in the chromatograms of the *Sample solution* and *Standard solution*.

Acceptance criteria: 0.2 µg/daily intake: The peak area for methylmercury in the chromatogram of the *Sample solution* is NMT the peak area for methylmercury in the chromatogram of the *Standard solution*.

⟨2250⟩ DETECTION OF IRRADIATED DIETARY SUPPLEMENTS

INTRODUCTION

Federal regulations do not permit irradiation of dietary ingredients or dietary supplements for sanitation purposes. Under section 201(s) of the Federal Food, Drug, and Cosmetic Act [21 USC 321(s)], irradiation is considered an additive, and as such it requires FDA approval. Foods that are irradiated should be adequately labeled according to international and national guidelines with statements such as "Treated with radiation" or "Treated by irradiation" in addition to information required by other regulations, including the irradiation logo, the Radura [21 USC 321(s)]. Overexposure to irradiation may have negative effects on product quality. Currently, several independent methods are used to identify irradiated foodstuffs, including dietary supplements. These methods have been validated and are recognized worldwide.

Procedures based on luminescence are widely applied and include screening by photostimulated luminescence (PSL), which is a rapid, simple preliminary screening method to detect irradiation, and a subsequent thermoluminescence (TL) procedure to confirm that the sample has been irradiated. PSL is less time consuming than TL because the inorganic/silicate mineral source of luminescence does not need to be isolated from any organic components present. Both PSL and TL signal intensities are affected by irradiation dose as well as by the nature and amount of inorganic material. TL analysis is one of the detection methods used for confirming the presence of irradiated foods, herbs, spices, vegetables, and fruits, although it has certain limitations: for example, samples must contain sufficient amounts of silicates that can be successfully separated from the samples. The lengthy preparation and need for irradiation in all cases limits TL to a small number of laboratories.

The procedures described in this chapter can be used both by regulatory authorities and by producers and suppliers of foods, including dietary supplements, to detect undeclared irradiated products in the market for purposes of determining compliance with regulations.

PRINCIPLES OF PHOTOSTIMULATED LUMINESCENCE AND THERMOLUMINESCENCE

Most of the natural dietary ingredients that are either cultivated or wild contain silicate minerals, calcite, or hydroxyapatite. Exposure to ionizing radiation from gamma rays (^{60}Co or ^{137}Cs), electron beams (up to 10 MeV), or x-rays (up to 5 MeV) stimulates electrons in those types of crystals, resulting in energized electrons being stored in the crystal lattice. Trapped high-energy electrons can be released by stimulation with light or controlled heat, leaving electron holes in the crystal lattice. The energy thus released is detected as luminescence. PSL occurs if light is applied to the system, and TL occurs when heat is applied to the system. The recorded luminescence intensity is proportional to the initial radiation dose absorbed.

Samples for both PSL and TL measurements must be taken from a light-protected position of the bulk samples, and exposure to high temperature and light must be avoided after the samples are taken from the bulk. Sample preparation and subsequent PSL or TL measurement must be conducted under subdued lighting conditions when possible.

TL has been widely used for detection of irradiation in food and dietary ingredients from which silicate minerals can be isolated. In this chapter, PSL and TL are described as the most appropriate detection methods for the materials typically used as dietary ingredients. The two methods are both radiation-specific phenomena, based on the observation that photon counts

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of irradiated samples usually are higher than those of nonirradiated samples. However, all available methods have some limitations in terms of their specific application range, product-to-product variation, complexity of or interference from the food matrix, and low concentration of radiation-induced markers.

GENERAL PROCEDURE FOR DETECTION OF IRRADIATION IN DIETARY INGREDIENTS AND DIETARY SUPPLEMENTS

Screening Using Photostimulated Luminescence

PSL, also known as optically stimulated luminescence, is based on the emission of light in the 300–600 nm range from irradiated samples when illuminated at longer wavelengths in the near-IR region. PSL analysis allows multiple measurements to be performed without sample pretreatment in a short period of time. PSL is also a nondestructive method that does not require separation of inorganic minerals and organic compounds. PSL is based on optical stimulation of mineral debris, typically silicates, and bioinorganic materials such as calcite, feldspar, or hydroxyapatite, and its sensitivity depends on the quantities and types of minerals present in the sample. Before the measurement of PSL, two thresholds are set: the lower typically is set at 700 counts/min (T1), and the upper typically is set at 5000 counts/min (T2). These two thresholds serve to classify the samples. After initial screening of PSL intensity from the samples is performed, the results are classified into negative (counts/ min less than T1), intermediate (counts/min NLT T1 and NMT T2), or positive (counts/min more than T2). A second measurement after a known irradiation dose, known as calibrated PSL (CalPSL), is applied in cases of poorly defined sample matrices whose luminescence sensitivities are not well established. By comparing the screening PSL measurement against its CalPSL response, analysts can take into account variation in detection sensitivity due to variable amounts and different types of minerals present in a given sample. CalPSL measurements are recommended to rule out false negative results due to low mineral content.

INSTRUMENTATION

The PSL system consists of pulsed IR sources for photostimulation, a single-photon counting system for highly sensitive detection of luminescence, a sample chamber, and a computer for data analysis.

The instrumental setup procedure includes checks of irradiated and nonirradiated materials, as well as establishing measurement parameters (cycle time, thresholds, and data-recording conditions). Measurement of initial background counts and periodic measurement of counts in the empty chamber should be conducted in subdued lighting to confirm lack of instrument contamination. The PSL signals (photon counts) emitted from the sample/second are automatically accumulated in the computer and are reported as counts/min. [NOTE—All experiments should be conducted under subdued lighting. In order to minimize the risk of cross-contamination, all preparations are performed in a laminar-flow cabinet.]

PHOTOSTIMULATED LUMINESCENCE MEASUREMENT

Procedure

PSL screening—Dispense and weigh two portions of the sample (5–10 g, depending on the density of the product) into two separate 50-mm disposable Petri dishes to cover the Petri base in a thin layer. Dispense samples in subdued lighting to minimize bleaching and under a laminar-flow cabinet to minimize cross-contamination. Measure the PSL of the sample for 1 min on the duplicate aliquots, and calculate the mean.

CALIBRATED PSL (CALPSL)— [NOTE—CalPSL testing may be done only once as part of the validation of the procedure for each material.]

Dispense and weigh two portions of the sample (5–10 g, depending on the density of the product) into 50-mm disposable Petri dishes to cover the Petri base in a thin layer. Measure the PSL of the sample for 1 min on duplicate aliquots, and for each sample calculate the mean. Irradiate the sample to a known dose of 1 kGy after the initial screening. Repeat the PSL measurement (CalPSL).

Evaluation—Classify results in the following manner:

<T1: negative, no evidence of PSL

>T1 and <T2: intermediate, weak PSL signal

>T2: positive, stronger signal.

ACCEPTANCE CRITERIA—Nonirradiated samples produce negative results in the PSL screening and are known to produce positive results in the CalPSL. [NOTE—Negative signals in the PSL screening are generally associated with nonirradiated material but can result from low-sensitivity irradiated materials. To evaluate whether a sample is a low-sensitivity material, assessment of the signals using the CalPSL option is necessary. Positive signals in the PSL screening are associated with irradiated material. Samples that are classified as intermediate require further investigation by the TL method to determine their irradiation status.]

Confirmatory Analysis Using Thermoluminescence

The TL analysis is based on physical changes in silicate minerals that are present in many food samples and dietary ingredients. The silicate minerals are able to store the absorbed radiation energy. A TL reader measures the amount of light that is emitted during controlled heating. The TL of the sample (TL_1) is compared to that of the same sample following irradiation at 1 kGy (TL_2). If the TL ratio (TL_1/TL_2) is greater than 0.1, the sample is considered to be irradiated. In order to use the TL method for the detection of irradiated foods or dietary ingredients, silicate minerals must be isolated from the samples.

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INSTRUMENTATION

TL measurements can be performed with a TL detector that meets the specifications in *Table 1*.

Table 1. Thermoluminesence Detector Specifications[a]

Radiation[b]	Photon: energies >5 keV Neutron: thermal to 100 MeV Electron/beta: energies >70 keV
Measurement ranges	10 µGy to 1 Gy (1 mrad to 100 rad) linear 1 Gy to 20 Gy (100 rad to 2000 rad) supralinear
Repeatability	For 1 mGy (100 mrad) ^{137}Cs doses, <2% standard deviation of 10 sequential measurements
Heating plate	50° to approximately 500°, approximately 6°/s

[a] Harshaw TLD 3500 (Thermo Fisher Scientific, Waltham, MA) and Nanogray TL2000 (Nanogray, Osaka, Japan) are suitable.
[b] Gamma rays from ^{60}Co or 10-MeV electron beams are suitable radiation sources.

PROCEDURE FOR SAMPLE PREPARATION

Sodium polytungstate solution: Prepare a solution of sodium polytungstate in water with a final density of 2 g/mL.
Mineral extraction: Suspend 3–20 g of sample with 50–100 mL of pure water, and sonicate for about 5 min. Sieve through a 250-µm nylon mesh, rinsing the mesh with water each time by using a strong jet from a wash bottle into a larger beaker (500–1000 mL). Allow the minerals to settle for 5 min and decant most of the water, leaving the minerals in <50 mL of water. Transfer the mineral fraction to a centrifuge tube, and centrifuge for 1 min at 1000 × g. Discard the water layer.
Preconcentration density separation: Add 5 mL of the *Sodium polytungstate solution* to the minerals in the centrifuge tube. Shake or mix on a vortex mixer, then sonicate for 5–15 min. Centrifuge for 2 min at 1000 × g. Silicate minerals (density 2.5–2.7 g/mL) precipitate, whereas organic components float. Carefully overlay a layer of water on the *Sodium polytungstate solution*, and discard the water layer and the organic materials by decantation or vacuum suction, leaving the minerals behind in the polytungstate layer. Carefully remove the *Sodium polytungstate solution* layer, and wash the minerals twice with water (centrifuge at 1000 × g briefly). Add 1–2 mL of 1 M hydrochloric acid, shake, and leave for 10 min in the dark to dissolve carbonates adhering to the silicate materials. Neutralize the acid with 1 M ammonium hydroxide. Fill the tube with water. Allow the minerals to settle, and centrifuge. Discard the water, and wash the minerals twice with water.
Fixing the minerals on disc for TL measurement: Add 3 mL of acetone to the preconcentrated minerals, and shake to displace residual water. Use a stainless steel disc suitable for the TL reader in use. [NOTE—Discs typically are 9–10 mm in diameter and 0.25–0.50 mm in thickness] . Carefully clean the disc by rinsing in water, wash two to three times with acetone, and dry in an oven. Store under dust-free conditions. Record the weight of the clean disc immediately before use. Transfer the minerals (in acetone), and dry the disc at 50° overnight (lab oven). Weigh the disc, and calculate the mass of minerals. Repeat the extraction, if necessary, to meet the system suitability requirements. [NOTE—The mineral sample amount is typically between 0.1 mg and 5 mg] . The deposited minerals can be fixed on the disc by using silicone spray (or by layering with 0.2% carboxymethylcellulose) and drying at 50° overnight.
Blank discs: Use clean discs.

TL MEASUREMENT

TL measurements are performed using a TL reader. Register the TL emission as a function of temperature (glow curves).
Minimum detectable integrated TL intensity level (MDL): Integrate the TL intensity of the first glow of the blank discs, and calculate the standard deviation. The MDL is three times the standard deviation of the integrated TL intensity of the blank discs.
System suitability: The integrated TL intensity of the irradiated sample (TL_2) should be at least 10 times the MDL.
Measurement of TL_1
First-glow TL_1 measurement—Set the instrument to an initial temperature of 70°, a heating rate of 6°/s, and a final temperature of 350°. Flush the chamber with nitrogen. Place the sample disc on the heating plate of the TL reader, and measure the glow curve. Determine TL_1 as the integrated TL signal between 150° and 250°. Measure the background glow for the sample (BG_1) after cooling the sample to 50°. After measuring TL_1 and BG_1, measure the weight of the sample (BW_1).
Irradiation—Irradiate the disc with the minerals, with a defined radiation dose of about 1 kGy. [NOTE—A source of ^{60}Co gamma rays is suitable.] Store at 50° overnight.
Measurement of TL_2
Second-glow TL_2 measurement—Measure the TL for the irradiated sample (TL_2) as described for TL_1. Measure the background glow for the irradiated sample (BG_2) after cooling the sample to 50°, and record the weight of the sample plate with the sample (BW_2). Measure the blank levels for process control (without the sample), following the procedures at each stage. Calculate the MDL as the integrated TL intensity of the blank plus three standard deviations.
TL glow ratio: The TL glow ratio per sample weight is calculated as described below:

$$\text{TL glow ratio} = [(TL_1 - BG_1)/BW_1]/[(TL_2 - BG_2)/BW_2]$$

TL_1 = irradiated sample for the first-glow measurement
BG_1 = background glow for the irradiated first-glow sample

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BW_1 = weight of the irradiated first-glow sample
TL_2 = irradiated sample for the second-glow measurement
BG_2 = background flow for the irradiated second-glow sample
BW_2 = weight of the irradiated second-glow sample

Evaluation: TL glow ratios from irradiated samples are typically greater than 0.1. The ratios from nonirradiated samples are below 0.1.

Acceptance criteria: The temperature that gives the maximum glow from TL_1 measurement must be equal to or higher than the one from a nonirradiated sample. The TL glow ratio must be NMT 0.1 for nonirradiated materials.

⟨2251⟩ SCREENING FOR UNDECLARED DRUGS AND DRUG ANALOGUES

INTRODUCTION

The illegal addition of undeclared synthetic compounds to products marketed as dietary supplements[1] (DS) is an issue of universal concern. This fraud is practiced to impart therapeutic effects that cannot be achieved by the dietary ingredients alone. Increasingly, synthetic intermediates and structural analogues of the pharmaceuticals and drugs that have been discontinued or withdrawn from the market are being used as adulterants. Multiple adulterating compounds may be added to a single product, frequently in erratic amounts.

The proposed test methodologies facilitate screening for synthetic adulterants. No individual technique is capable of addressing all potential analytes; thus, a combination of orthogonal approaches adds certainty to the analytical outcome. Mass spectrometric techniques provide strong substantiation of the analytical findings. In some cases, e.g., with hormonal drugs, the amounts of physiologically relevant adulterants may be so low that GC-MS or LC-MS may be the only fitting analytical options.

The express purpose of assembling the procedures recommended herein is their suitability for screening. The level of evidence achievable by application of one or several of the recommended procedures is ultimately dictated by the specific requirements of the end-user. It should be noted that structure elucidation and quantitative assessment are beyond the scope of this chapter.

This chapter is meant to be updated regularly, as new concealment methodologies for the adulterants are introduced, or improvements to the methods of analysis are realized.

ADULTERATION CATEGORIES

The following major categories of adulterated products are recognized:

- **Sexual Enhancement:** This category is also referred to as the Erectile Dysfunction (ED) category. It encompasses a functionally coherent group of adulterants, including several approved drugs, their numerous approved and unapproved analogues, and synthetic intermediates. Their functionality is manifested by inhibition of phosphodiesterase type 5 enzyme (PDE5), which hydrolyzes cyclic guanosine 3′,5′-monophosphate (cGMP); this group of compounds is frequently identified as PDE5 inhibitors. Screening methods for products adulterated with ED compounds are presented in *Appendix A*.
- **Weight Loss (WL):** This category comprises a functionally and chemically diverse collection of compounds that include stimulants, laxatives, diuretics, anorexiants, and psychoactive drugs. Although stimulants constitute an important segment of WL adulterants, the oral anorexiant sibutramine dominates this category, frequently in combination with phenolphthalein, a laxative. Methods for analysis of products adulterated with WL compounds will be addressed in *Appendix B* (to come).
- **Sports Performance Enhancement (SPE):** These compounds constitute the third major category of adulteration. Professional and amateur athletes are targeted with designer anabolic steroids and stimulants, which are systematically banned by the World Anti-Doping Agency. Functional and structural diversity, synthetic proclivity of the adulterators, and the generally small amounts of the infringing substances required to elicit a therapeutic effect make this category especially challenging to address. These supplements are customarily formulated in protein- and fat-rich matrices, thereby further complicating detection. For these reasons, GC- and $LC\text{-}MS^n$ techniques constitute primary analytical methodologies within this category. Analysis of products adulterated with SPE compounds will be addressed in *Appendix C* (to come).

BULK INGREDIENTS AND DOSAGE FORMS

Adulteration may occur either at the level of bulk ingredients or at any subsequent stage of the finished product manufacturing. Analysts should be mindful of the possibility of adulterants physically associated with the finished dosage matrix or excipients, as well as components. In the latter, synthetic compounds have been found embedded into the capsule shell body. This underscores the need for deliberate adjustment to the laboratory procedures that typically focus on the capsule contents alone. Appropriate sampling practices for powders and finished dosage forms should be exercised, particularly when only a limited amount of sample is available.

[1] In the United States, dietary supplements are defined as substances that are ingested, in agreement with 21 U.S. Code §321(ff)(2)(A)(i). Definitions of dietary supplements, nutritional supplements, functional foods, and bioactive food additives may vary extensively, depending on local or national legislation. In the marketplace, there is a trend toward expanding the mode of delivery of the adulterating compounds to routes not covered by the regulatory definition for dietary supplements, i.e., topical oils, creams, lotions, e-cigarettes, chewing gums, sprays, and others. Such novel delivery systems present unique challenges, particularly from the standpoint of sample preparation, and are not considered for the purposes of this chapter to be dietary supplements. However, recognizing the emerging threat, USP chooses to highlight the existence of these products. In no way should mention of these products be interpreted as a comment on their legal status or be perceived as an expansion of the definition of DS.

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RECOMMENDED ANALYTICAL METHODOLOGIES

Adulteration analysis may be broadly categorized into targeted and nontargeted methods. The distinction between these types may be subtle, and a minor adjustment to the methodology will transform a nontargeted method into a targeted method.

Targeted

These techniques are warranted when the analytes are known. An example of a targeted approach would be monitoring a chromatographic run at a particular wavelength (or mass), and quantifying the analyte that appears within a predefined retention time window. Targeted analysis is conceptually straightforward, because it relies on pre-existing knowledge of the analyte and allows optimization of test methodology for its reliable detection. The targeted approach also is a rarity in the adulterated products analysis, where the nature of the analyte may be anticipated only tentatively, and variable amounts of multiple adulterants belonging to several functional categories are commonplace.

Nontargeted

These methods are better suited to a broad-spectrum detection requirement presented by adulterated products. Nontargeted screening trades precise knowledge of the analyte identity, along with specificity and accuracy, for a wider detection scope. Examples of nontargeted chromatographic screening include acquisition of photodiode array data and full mass-spectral scanning following a chromatographic separation. The procedures in this chapter are written with an eye toward applying all techniques in a nontargeted mode, even the ones considered to be inherently targeted, thereby facilitating detection of a suspect adulterant even in the absence of a matching reference compound.

It is generally recommended to apply a broadly nontargeted methodology first, followed by a targeted procedure. It is crucial to clearly define the end-purpose of analysis, and only then decide on the appropriate instrumentation and assemble a logical testing strategy from the procedures provided. Thus, application of a nontargeted screening method may satisfy the requirements of a manufacturer for the purposes of monitoring bulk raw materials. Conversely, a laboratory requiring a higher level of evidence to enact an enforcement action may opt for a two-step procedure: a preliminary screen, followed by confirmatory analysis of suspect samples.

USP Reference Standards recommended for screening are listed at the end of each relevant *Appendix*. However, considering the rate of propagation of structural analogues and proliferation of newly developed "designer" molecules, establishing and maintaining an all-inclusive catalog of reference materials is both challenging and impractical. Several commercial sources of the compounds of interest exist.[2] Please note that mention of the external reference materials suppliers does not in any way constitute their endorsement, as neither does the listing of reagents, supplies, and instrumentation.

APPENDIX

• APPENDIX A. SCREENING METHODOLOGIES FOR PDE5 INHIBITORS

1. HPLC with Photodiode Array Detection

Solution A: 0.1% Formic acid in water

Solution B: 0.1% Formic acid in acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
15	5	95
23	5	95
24	95	5
31	95	5

Diluent: Acetonitrile and water (50:50)

Standard solution: 100 µg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in *Diluent*

Sample solution: Combine one-fifth of the dosage unit, 10–20 mg of bulk material, or a small fragment of the capsule shell (3 mm × 3 mm) with 10 mL of *Diluent*, sonicate for 30 min, and pass through a 0.2-µm PTFE syringe filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Photodiode array, 200–400 nm

Analytical wavelength: 290 nm

Column: 2.1-mm × 15-cm; 5-µm packing L1[3]

Column temperature: 40°

Flow rate: 0.2 mL/min

Injection volume: 1 µL

System suitability

Sample: *Standard solution*

[2] CacheSyn (http://www.cachesyn.com/); Santa Cruz Biotechnology, Inc. (http://www.scbt.com); TLC Pharmachem (http://www.tlcpharmachem.com/); and Toronto Research Chemicals (http://www.trc-canada.com/) are some of the potential sources of rare and hard-to-find adulterant reference materials.

[3] The procedure was developed on the Agilent Technologies Zorbax SB-C18 column.

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Suitability requirements
Column efficiency: NLT 3000 theoretical plates
Tailing factor: NMT 1.5
Analysis
Sample: *Sample solution*
Examine the UV spectra of the prominent peaks for similarity to those in the *Standard solution* or other known PDE5 inhibitor compounds (*Figure 1* and *Table 5*). Typical retention times of several PDE5 inhibitors are provided in *Table 5*. However, neither retention time match nor the absorbance spectrum similarity should be construed as sufficient confirmation of the chemical identity of an adulterant.

2. HPLC with Mass-Spectrometric Detection
Preferably, a mass-spectrometric detector is connected in sequence to the UV-Vis detector. The settings below apply to an ion-trap mass spectrometer. Other MS detectors are suitable; however, it is advisable to use spectrometers that possess MS/MS capability.
Solution A: 0.1% Formic acid in water
Solution B: 0.1% Formic acid in acetonitrile
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	95	5
15	5	95
23	5	95
24	95	5
31	95	5

Diluent: Acetonitrile and water (50:50)
Standard solution: 5 µg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in *Diluent*
Sample solution: Combine one-fifth portion of the dosage unit, 10–20 mg of bulk material, or a small fragment of the capsule shell (3 mm × 3 mm) with 10 mL of *Diluent*, sonicate for 30 min, and pass through the 0.2-µm PTFE syringe filter. Dilute the filtrate 20-fold with *Diluent* before injection.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 290 nm
Column: 2.1-mm × 15-cm; 5-µm packing L1[4]
Column temperature: 40°
Flow rate: 0.2 mL/min
Injection volume: 1 µL
Mass spectrometric system[5]
(See *Mass Spectrometry* ⟨736⟩.)
Ionization: ESI
Polarity: Positive or negative
Sheath gas: 35 mL/min
Sweep gas: 5 mL/min
Capillary temperature: 300°
Source voltage: 5 kV
Collision: 45 meV
Scanning: *m/z* 90–1050 and dependent scan on the most intense ion
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 2.0 between vardenafil and sildenafil peaks
Tailing factor: NMT 1.5
Analysis
Sample: *Sample solution*
Compare mass-to-charge ratios of the molecular ions $[M+H]^+$ or $[M-H]^-$ and fragments to those of the *Standard solution* or other known analytes listed in *Table 4*. Typical retention times of several common PDE5 inhibitors are provided in *Table 5*.

[4] The procedure was developed on the Agilent Technologies Zorbax SB-C18 column.
[5] The settings were found appropriate for ThermoElectron LTQ XL Linear Ion Trap Mass Spectrometer. Users will need to optimize their respective instrumentation according to the manufacturer's recommendations.

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3. High Performance Thin-Layer Chromatography (HPTLC) with Visual, UV, and/or MS Detection

Standard solution: A composite of 0.2-mg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in methanol, with sonication if necessary. Additional reference materials may be available commercially.

Sample solution: Comminute 1 dosage unit, including the capsule shell and tablet coating, or about 500 mg of raw material; combine with 10 mL of methanol, and subject to ultrasonication for 30 min. Centrifuge or filter the solution, and use the supernatant. [NOTE—Upon development, if the chromatographic bands appear too saturated and UV densitometric spectra are distorted, dilute the *Sample solution* 10-fold with methanol.]

Developing solvent system: *tert*-Butyl methyl ether, methanol, and 28.0% (w/w) ammonium hydroxide (20:2:1). [NOTE—Strength of ammonium hydroxide was found to be crucial for adequate method performance. It is therefore advisable to establish the titer of higher-concentration ammonia[6] and to adjust the latter to exactly 28.0% immediately before the experiment.]

Chromatographic system

(See *HPTLC for Articles of Botanical Origin* ⟨203⟩.)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm

Application volume: 3 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 47% using a suitable device in the presence of a saturated solution of potassium isothiocyanate.

Temperature: Ambient

Saturation: 20 min, with paper

Developing distance: 6 cm

Derivatization reagent: None

Drying: 5 min in a current of cold air

Detection 1: Visual, under illumination with 254- and 365-nm UV light

Detection 2: UV-Vis spectrometry (scanning densitometer), 190–550 nm

Detection 3: Mass spectrometry, *m/z* 90–1050

Mass spectrometric system[7]

(See *Mass Spectrometry* ⟨736⟩.)

Ionization: ESI

Polarity: Positive, negative, or rapid switching

Desolvation gas (N_2): 300 L/h

Cone gas (N_2): 80 L/h

Temperatures

ESI probe: 105°

Desolvation: 150°

Capillary voltage: 3.0 kV

Cone voltage: 50 V

Scanning: *m/z* 90–1050

System suitability

Sample: *Standard solution*

Suitability requirements: Under UV light at 254 nm, sildenafil, tadalafil, and vardenafil appear as dark bands against the fluorescent background. Under UV light at 365 nm, sildenafil, tadalafil, and vardenafil appear as blue fluorescent bands.

Analysis: Inspect the plate under short-wave (254 nm) and long-wave (365 nm) UV light. PDE5 inhibitors appear as dark bands against the fluorescent background at 254 nm and typically exhibit different shades of blue fluorescence under 365 nm. Note the similarities in R_F values between the bands in the *Standard solution* and *Sample solution*; these may be informative, however they do not constitute sufficient proof of identity. Relative intensities of the bands permit approximation of the amounts. Using scanning densitometry, obtain UV spectra of the prominent bands in the *Sample solution*, and compare them to those of the PDE5 inhibitors in the *Standard solution* and those provided in *Table 5* and *Figure 1*. Mass-spectrometric interface, if available, may facilitate more definitive assignment of the analyte bands: compare mass-to-charge ratios of the molecular ions $[M+H]^+$ or $[M-H]^-$ and fragments to those of the common adulterants listed in *Table 4*.

4. Ambient Ionization Mass Spectrometry

Diluent: Acetonitrile and water (50:50), with 0.1% formic acid

Standard solution: A composite solution containing 20 µg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in *Diluent*. Additional reference materials may be available commercially.

Sample solution: Grind the entire dosage form, including the capsule shell and tablet coating, to a fine powder. Weigh about 50 mg of the resulting powder, or about 50 mg of bulk material, and combine with 5 mL of *Diluent*. Cap tightly, subject to ultrasonication for 2 min, and vortex thoroughly. Centrifuge or filter the resulting solution, and dilute an aliquot of the supernatant or filtrate 100-fold with *Diluent*.

Mass spectrometric system

(See *Applications of Mass Spectrometry* ⟨1736⟩, *Mass Spectrometers, Ionization Procedures, Ambient Ionization Procedures*.)

Ionization: Ambient with thermal desorption

[6] 32% Ammonia solution is available from EMD Millipore.

[7] Procedure was developed using Expression CMS mass spectrometer from Advion, equipped with a TLC-MS interface available from CAMAG. If other mass spectrometers are used, relevant settings will have to be optimized. The bands were directly eluted with a mixture of water and acetonitrile (80:20) containing 0.1% formic acid.

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Mode: Thermal profile
Polarity: Positive, negative, or rapid switching
Gas temperature: 150°, 250°, 350°, and 450°
Scanning: *m/z* 90–1050

System suitability: Deposit 3-µL aliquots of the *Standard solution* onto the disposable sample cards. Set the compatible mass spectrometer to a 30-s acquisition of 90–1050 Da. Using one sample card for each temperature setting, acquire mass spectra at each of the following desorption gas temperatures: 150°, 250°, 350°, and 450° in positive ionization mode. Switch polarity, and re-acquire spectra at the same four temperatures in the negative ionization mode. [NOTE—If the mass spectrometer permits rapid polarity switching, both positive and negative spectra may be acquired simultaneously using a single sample.] Confirm that the $[M+H]^+$ or $[M-H]^-$ ions of sildenafil, tadalafil, and vardenafil are observed as listed in *Table 4*.

Analysis: Deposit 3-µL aliquots of the *Sample solution* onto the disposable sample cards, and follow the procedure outlined above for the *Standard solution*. In the event that a single ion dominates the mass spectrum at every temperature setting, dilute the *Sample solutions* 10-fold with *Diluent* and re-analyze. Compare mass-to-charge ratios of the molecular ions $[M+H]^+$ or $[M-H]^-$ and fragments to those of the known analytes listed in *Table 4*.

5. **NMR Spectroscopy–Low-Field and High-Field**

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis*.) [NOTE—Deuterated acetonitrile (CD_3CN) should be NLT 99.8 atom % D, and should contain 0.05% tetramethylsilane (TMS) as a chemical shift reference. Use of solvents in sealed ampules is recommended. NMR tubes should be suitable for use at the selected magnetic field strength.]

Standard solutions: Dissolve 10 mg of USP Sildenafil Citrate RS, USP Tadalafil RS, or USP Vardenafil Hydrochloride RS in separate 1-mL aliquots of CD_3CN, and transfer 700-µL aliquots of the resulting solutions into individual NMR tubes. Additional reference materials are available commercially.

Sample solution: Grind the entire dosage form, including the capsule shell and tablet coating, to a fine powder. Transfer 100–200 mg of the ground material, or an equivalent amount of bulk raw material powder, into a 5-mL sealable glass vial. Add 1 mL of CD_3CN, vortex thoroughly, and allow the solids to settle. Transfer about 700 µL of the supernatant to an NMR tube, taking care to minimize transfer of solids.

Instrument performance qualification

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.)

Magnetic field strength: NLT 42.5 MHz (^1H operating frequency)

Data collection: Use the parameters specified in *Table 3*; perform 90° pulse width calibration before the analysis according to the recommendations of the equipment manufacturer.

Table 3

Parameter	^1H-NMR Qualitative Measurement
Pulse program	Single pulse ^1H
Spectral width	14 ppm (–1 to 13 ppm)
Transmitter offset	Center of spectral width
Relaxation delay	5–10 s
Acquisition time	2–5 s
Number of data points per FID[a]	NLT 16,000
Temperature	25°

[a] Free induction decay.

System suitability: Acquire a ^1H spectrum of the *Standard solution* using the settings outlined in *Data collection*. Record a sufficient number of scans to ensure that signal-to-noise ratio of the TMS signal is NLT 10.

Analysis: Acquire a ^1H spectrum of the *Sample solution* under the conditions outlined in *Data collection*. Record a sufficient number of scans to ensure that the signal-to-noise ratio of the TMS signal is NLT 10. Reference all acquired spectra to the ^1H signal of TMS (0 ppm). Measure and record the chemical shift and multiplicity of the NMR signals in the spectra of the *Standard solutions* and *Sample solution*. Compare the ^1H NMR spectrum of the *Sample solution* to those of the *Standard solutions*, paying particular attention to the aromatic region (5–9 ppm). Determine whether the chemical shift and multiplicity of the NMR signals in the *Sample solution* exhibit sufficient similarity to those found in the *Standard solutions*.

6. **Bioassay**[8]

PDE5 enzyme[9] **stock solution:** Prepare a concentration of approximately 3000 Units/µL. If necessary, dilute with 40 mM tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl), pH 8.0, 110 mM sodium chloride (NaCl), 2.2 mM potassium chloride (KCl), 3 mM dithiothreitol, and 20% glycerin. Vortex gently to mix.

[8] The procedures were developed using commercial Promega PDE-Glo™ Phosphodiesterase Assay Kit, Catalog # V1361. It includes the following reagents: PDE-Glo™ Reaction Buffer 5X (Catalog # V133A); PDE-Glo™ Detection Buffer 5X (Catalog # V134A); Protein Kinase A Solution (Catalog # V135A); PDE-Glo™ Termination Buffer 5X (Catalog # V136A); cGMP Stock Solution, 1 mM (Catalog # V641A); cAMP, 1 mM (Catalog # V642B); Kinase-Glo™ Substrate (Catalog # V672A); and Kinase-Glo™ Buffer (Catalog # V673A). Kits from alternative suppliers may also be used, e.g., BPS Science, Catalog # 60350, although re-optimization of test procedures will be required.

[9] The procedures were developed using human phosphodiesterase 5A from BPS Bioscience Catalog # 60050. The enzyme is available from numerous suppliers, e.g., Sigma-Aldrich Catalog # E9034.

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PDE5 working solution (100 Units per 6.5 µL): Combine 400 µL of PDE-Glo™ Reaction Buffer 5X, 10 µL of *PDE5 enzyme stock solution*, and 1590 µL of water. Vortex gently to mix.
cGMP solution: Combine 400 µL of PDE-Glo™ Reaction Buffer 5X, 40 µL of 1-mM cGMP stock solution, and 1560 µL of water. Mix thoroughly by vortexing.
100-mM IBMX stock solution in DMSO: Prepare a 22.2-mg/mL solution of 3-isobutyl-1-methylxanthine (IBMX) in dimethylsulfoxide (DMSO), e.g., dissolve 100 mg of IBMX in 4.5 mL of DMSO. Mix thoroughly by vortexing.
Reaction buffer: Combine 400 µL of PDE-Glo™ Reaction Buffer 5X and 1600 µL of water. Mix thoroughly by vortexing.
Reaction buffer with 4% DMSO: Combine 400 µL of PDE-Glo™ Reaction Buffer 5X, 80 µL of DMSO and 1520 µL of water. Mix thoroughly by vortexing.
Termination buffer: Combine 400 µL of PDE-Glo™ Termination Buffer 5X, 40 µL of *100-mM IBMX stock solution in DMSO*, and 1560 µL of water. Mix thoroughly by vortexing.
Detection buffer: Combine 400 µL of PDE-Glo™ Detection Buffer 5X, 16 µL of Protein Kinase A Solution, and 1584 µL of water. Vortex gently to mix.
Kinase-Glo™ reagent: Add 10 mL of Kinase-Glo™ Buffer to the vial of Kinase-Glo™ Substrate, and vortex gently.
Standard solution (400 nM): Dissolve 5 mg of USP Sildenafil Citrate RS in 3.0 mL of DMSO to obtain a 2.5-mM stock solution. Combine a 10-µL aliquot of the resulting solution with 240 µL of DMSO, and mix thoroughly (100 µM). Combine a 10-µL aliquot of the resulting solution with 90 µL of DMSO, and mix thoroughly (10 µM). Combine a 10-µL aliquot of the resulting solution with 240 µL of *Reaction buffer*, and mix thoroughly (400 nM); the last dilution is the *Standard solution*.
Control solution: Combine 10 µL of DMSO with 240 µL of *Reaction buffer*, and mix thoroughly by vortexing.
Sample solution: Grind the entire dosage form, including the capsule shell and tablet coating, to a fine powder. Transfer 100 mg of the ground material into a 5-mL polypropylene vial. Add 3.0 mL of DMSO, and vortex for 60 s. Allow solids to settle, combine 50 µL of the clear supernatant with 200 µL of DMSO, and mix thoroughly by vortexing. Combine a 10-µL aliquot of the resulting solution with 90 µL of DMSO, and mix thoroughly by vortexing. Combine a 10-µL aliquot of the resulting solution with 240 µL of *Reaction buffer*, and mix thoroughly by vortexing; the last dilution is the *Sample solution*.

Analysis

1. Dispense 12.5-µL aliquots of the *Standard solution*, *Control solution*, and *Sample solution* into microplate wells, in triplicate. Use a white, flat-bottom, opaque polystyrene, nontreated, 96-well microtiter plate.[10] [NOTE—Do not use treated plates, black plates, or clear plates.]
2. Add 6.5 µL of *PDE5 working solution* to each well. Incubate for 5 min.
3. Add 6.0 µL of *cGMP solution* to effect a 5-µM cGMP concentration in a 25-µL volume. Incubate for 30 min.
4. Add 12.5 µL of *Termination buffer*. Incubate for 5 min.
5. Add 12.5 µL of *Detection buffer*. Incubate for 20 min.
6. Add 50 µL of *Kinase-Glo™ reagent*. Incubate for 10 min.
7. Record luminescence at 560 nm with a microplate luminometer at 0.5 s/well.

[NOTE—Incubate the plate at room temperature, preferably using a plate shaker.]

Calculate average luminescence values for the replicate preparations. Assess the degree of PDE5 inhibition in the *Sample solution* relative to that observed in the *Standard solution* and *Control solution*. Inhibition of PDE5 is manifested as reduction of luminescence: the samples that exhibit suppression of luminescent output comparable to or in excess of that observed with the *Standard solution* are likely adulterated with synthetic PDE5 inhibitors.

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Sildenafil Citrate RS
  USP Tadalafil RS
  USP Vardenafil Hydrochloride RS

Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a]

#	Name	CAS Number	Chemical Formula	Exact Mass	$[M+H]^+$	$[M-H]^-$	Fragments
1	Acetaminotadalafil	1446144-71-3	$C_{23}H_{20}N_4O_5$	432.1434	433	—	455 $[M+Na]^+$, 391, 311, 269, 250
2	Acetil acid	147676-78-6	$C_{18}H_{20}N_4O_4$	356.1485	357	—	329, 300, 285, 268, 256, 242, 166, 131
3	Acetildenafil (Hongdenafil)	831217-01-7	$C_{25}H_{34}N_6O_3$	466.26924	467.28	—	449, 439, 420, 404, 396, 381, 355, 353, 339, 325, 324, 311, 297, 285, 166, 127, 111, 99, 97
4	Acetylvardenafil	1261351-28-3	$C_{25}H_{34}N_6O_3$	466.2692	467	—	439, 396, 341, 317, 270
5	Aildenafil (Dimethylsildenafil, Methisosildenafil)	496835-35-9	$C_{23}H_{32}N_6O_4S$	488.22057	489.23	—	432, 377, 313, 311, 283, 113, 99
					—	487.40	460, 310, 282

[10] Conforming plates are available from Corning (Costar 3912 or Costar 3963), Thermo Scientific (Nunc™ Catalog #236105), and other vendors.

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Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a] *(continued)*

#	Name	CAS Number	Chemical Formula	Exact Mass	$[M+H]^+$	$[M-H]^-$	Fragments
6	Aminotadalafil	385769-84-6	$C_{21}H_{18}N_4O_4$	390.1328	391.14 —	— 389.1248	269, 262, 241, 239, 224, 197, 169 362, 298, 262, 234, 233, 232
7	(*S,R*)-Aminotadalafil [(+)-*trans*-Aminotadalafil]	1093940-70-5	$C_{21}H_{18}N_4O_4$	390.1328	391	—	No data
8	Avanafil	330784-47-9	$C_{23}H_{26}N_7O_3Cl$	483.1786	484.186	—	375, 349, 221
9	Benzylsildenafil	1446089-82-2	$C_{28}H_{34}N_6O_4S$	550.2362	551	—	377, 283
10	*N*-Butylnortadalafil	171596-31-9	$C_{25}H_{25}N_3O_4$	431.18451	432.25	—	**310**,[b] *282*,[c] *197, 169*
11	Carbodenafil (Fondenafil)	944241-52-5	$C_{24}H_{32}N_6O_3$	452.2536	453	—	283
12	Chlorodenafil	1058653-74-9	$C_{19}H_{21}ClN_4O_3$	388.8485	390	—	360, 311, 291, 254, 183, 136
13	Chloropretadalafil	171489-59-1	$C_{22}H_{19}ClN_2O_5$	426.0982	427	—	429, 395, 349, 334, 302, 287, 262, 229, 159, 135
14	Cinnamyldenafil	1446089-83-3	$C_{32}H_{38}N_6O_3$	554.3005	554	—	488, 354, 297, 283, 215, 166, 117, 91
15	Cyclopentynafil	1173706-34-7	$C_{26}H_{36}N_6O_4S$	528.2519	529	—	461, 153
16	Depiperazinothiosildenafil	1353018-10-6	$C_{17}H_{20}N_4O_4S_2$	408.0926	409 —	— 407.0845	381, 351, 327, 299, 285, 272 378, 314
17	Descarbonsildenafil	1393816-99-3	$C_{21}H_{30}N_6O_4S$	462.2049	463	—	418, 377, 360, 311, 299, 283, 255, 151, 87
18	*N*-Desethylvardenafil	448184-46-1	$C_{21}H_{28}N_6O_4S$	460.18927	461.20	—	392, 377, 376, 329, 313, 312, 299, 284, 283, 151
19	Desmethylcarbodenafil	147676-79-7	$C_{23}H_{31}N_6O_3$	438.523	439.2451	—	339, 311
20	*N*-Desmethylsildenafil	139755-82-1	$C_{21}H_{28}N_6O_4S$	460.18927	461.19	—	377, 313, 311, 299, 283
21	Dimethylacetildenafil	1417999-76-8	$C_{25}H_{34}O_3N_6$	466.2771	467	—	279, 149, 177
22	Dioxo-acetildenafil	1609405-33-5	$C_{25}H_{30}N_6O_5$	494.2278	495	—	No data
23	Dithio-desmethylcarbodenafil	1333233-46-7	$C_{23}H_{30}N_6OS_2$	470.6572	471.1991	—	371, 343
24	Gendenafil	147676-66-2	$C_{19}H_{22}N_4O_3$	354.16919	355.31	—	327, 298, **285**, 283, *256, 242*
25	Gisadenafil	334826-98-1	$C_{23}H_{33}N_7O_3S$	519.22639	520	—	No data
26	Homosildenafil	642928-07-2	$C_{23}H_{32}N_6O_4S$	488.22057	489.23	—	467, 461, 377, 313, 311, 283, 127, 111, 113, 99, 97
27	Hydroxyacetildenafil (Hydroxyhongdenafil)	147676-56-0	$C_{25}H_{34}N_6O_4$	482.26415	483.27	—	465, 447, 439, 396, 353, 339, 325, 311, 297
28	Hydroxychlorodenafil	1391054-00-4	$C_{19}H_{23}ClN_4O_3$	390.1459	391	—	No data
29	Hydroxyhomosildenafil	139755-85-4	$C_{23}H_{32}N_6O_5S$	504.21549	505.22 —	— 503.31	487, 461, 423, 377, 312, 311, 283, 284, 225, 166, 129, 112, 99, 97 475, 310, 282
30	2-Hydroxypropylnortadalafil	1353020-85-5	$C_{24}H_{23}N_3O_5$	433.16377	434.15	—	**312**, *284, 197, 169*
31	Hydroxythiohomosildenafil (Hydroxyhomosildenafil thione, Sulfohydroxyhomosildenafil)	479073-82-0	$C_{23}H_{32}N_6O_4S_2$	520.19264	521.20	—	503, 477, 461, 419, 393, 355, 354, 327, 325, 291
32	Hydroxythiovardenafil	912576-30-8	$C_{23}H_{32}N_6O_4S_2$	520.1926	521	—	No data

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Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a] *(continued)*

#	Name	CAS Number	Chemical Formula	Exact Mass	$[M+H]^+$	$[M-H]^-$	Fragments
33	Hydroxyvardenafil	224785-98-2	$C_{23}H_{32}N_6O_5S$	504.2155	505.26 —	— 503	344, 312, 253, 169, 99 475, 310, 282
34	Imidazosagatriazinone (Desulfovardenafil)	139756-21-1	$C_{17}H_{20}N_4O_2$	312.1586	313	—	284, 256, 169, 151
35	Isopiperazinonafil	1335201-06-3	$C_{25}H_{34}N_6O_4$	482.2642	483 —	— 481	— 453, 422, 379, 336, 325, 311, 309
36	Lodenafil carbonate	398507-55-6	$C_{47}H_{62}N_{12}O_{11}S_2$	1034.4102	1035	—	518, **487**, *377*, *311*
37	Mirodenafil	862189-95-5	$C_{26}H_{37}N_5O_5S$	531.6698	532	—	488, 404, 362, 296, 268
38	Mutaprodenafil	1387577-30-1	$C_{27}H_{35}N_9O_5S_2$	629.7635	630.2279	—	489, 377, 142, 113
39	Nitrodenafil	147676-99-1	$C_{17}H_{19}N_5O_4$	357.3647	358	—	307, 289, 261, 217, 176, 154, 136, 107, 89
40	Nitroso-prodenafil	1266755-08-1	$C_{27}H_{35}N_9O_5S_2$	629.2203	630	—	142
41	Noracetildenafil (Demethylhongdenafil)	949091-38-7	$C_{24}H_{32}N_6O_3$	452.25359	453.26	—	425, 406, 396, 380, 367, 355, 353, 339, 325, 324, 313, 297, 296, 253
42	Norneosildenafil (Piperidino sildenafil)	371959-09-0	$C_{22}H_{29}N_5O_4S$	459.19403	460.20	—	432, 377, 329, 311, 299, 283
43	Norneovardenafil	358390-39-3	$C_{18}H_{20}N_4O_4$	356.1485	357	—	329, 307, 289, 176, 154, 136, 107, 99
44	Nortadalafil (Demethyltadalafil)	171596-36-4	$C_{21}H_{17}N_3O_4$	375.1219	376	374.1138	262, 234, 233, 232
45	*N*-Octylnortadalafil (Octylnortadalafil)	1173706-35-8	$C_{29}H_{33}N_3O_4$	487.2471	488	—	366, 227
46	Oxohongdenafil	1446144-70-2	$C_{25}H_{32}N_6O_4$	480.2485	481	—	451, 396, 354, 339, 312, 297, 289
47	Piperazinonafil (Piperazonifil, Dihydroacetildenafil)	1335201-04-1	$C_{25}H_{34}N_6O_4$	482.2642	483 —	— 481	— 453, 435, 348, 336, 321, 311, 309, 282, 267
48	Piperidino acetildenafil (Piperiacetildenafil)	147676-50-4	$C_{24}H_{31}N_5O_3$	437.2427	438	—	410, 408, 355, 353, 341, 325, 297, 288
49	Piperidinovardenafil (Piperidenafil, Pseudovardenafil)	224788-34-5	$C_{22}H_{29}N_5O_4S$	459.19403	460.20	—	432, 403, 391, 377, 349, 329, 312, 311, 301, 299, 284, 283, 270, 256, 169, 151
50	Propoxyphenyl aildenafil	1391053-82-9	$C_{24}H_{34}N_6O_4S$	502.2362	503	—	252
51	Propoxyphenyl hydroxyhomosildenafil (Methylhydroxyhomosildenafil)	139755-87-6	$C_{24}H_{34}N_6O_5S$	518.2311	519	—	501, 475, 391, 331, 325, 299, 283, 129, 112, 99
52	Propoxyphenyl sildenafil	877777-10-1	$C_{23}H_{32}N_6O_4S$	488.2205	489.2272	—	447, 391, 325, 299, 283, 100
53	Propoxyphenyl thioaildenafil (Propoxyphenyl thiomethisosildenafil)	856190-49-3	$C_{24}H_{34}N_6O_3S_2$	518.2133	519	—	260
54	Propoxyphenyl thiohydroxyhomosildenafil	479073-90-0	$C_{24}H_{34}N_6O_4S_2$	534.2083	535.2150	—	517, 359, 341, 315, 299, 271, 129, 112, 99
55	Sildenafil	139755-83-2	$C_{22}H_{30}N_6O_4S$	474.20492	475.21 —	— 473.45	447, 418, 391, 377, 374, 346, 329, 311, 297, 283, 255, 163, 160, 100 445, 310, 282

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Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a] *(continued)*

#	Name	CAS Number	Chemical Formula	Exact Mass	$[M+H]^+$	$[M-H]^-$	Fragments
56	Tadalafil (Tildenafil)	171596-29-5	$C_{22}H_{19}N_3O_4$	389.13756	390 —	— 388.1288	302, 268, 262, 250, 240, 197, 169, 135 262, 234, 233, 232
57	(–)-*trans*-Tadalafil (ent-Tadalafil)	629652-72-8	$C_{22}H_{19}N_3O_4$	389.1376	390	—	779 $[2M+H]^+$, 262, 250, 135
58	Thioaildenafil (Sulfoaildenafil, Thiomethisosildenafil, Sulfodimethyl sildenafil, Dimethylthiosildenafil)	856190-47-1	$C_{23}H_{32}N_6O_3S_2$	504.19773	505.21	—	448, 393, 327, 299, 113, 99
59	Thiohomosildenafil (Sulfohomosildenafil, Homosildenafil thione)	479073-80-8	$C_{23}H_{32}N_6O_3S_2$	504.19773	505.21	—	477, 421, 393, 357, 355, 343, 327, 315, 299, 271, 113, 99
60	Thioquinapiperifil (KF31327)	220060-39-9	$C_{24}H_{28}N_6OS$	448.2045	449	—	363, 246, 225, 204, 121
61	Thiosildenafil (Sulfosildenafil, Sildenafil thione)	479073-79-5	$C_{22}H_{30}N_6O_3S_2$	490.18208	491.19	—	407, 393, 343, 341, 327, 315, 313, 299, 283, 271, 163, 99
62	Udenafil	268203-93-6	$C_{25}H_{36}N_6O_4S$	516.2519	517.260	—	474, 418, 347, 325, 299, 283
63	Vardenafil	224785-90-4	$C_{23}H_{32}N_6O_4S$	488.22057	489.2274 —	— 487.33	461, 420, 377, 376, 375, 346, 339, 329, 312, 299, 284, 283, 169, 151, 123, 99 459, 310, 282
64	Xanthoanthrafil (Benzamidenafil)	1020251-53-9	$C_{19}H_{23}N_3O_6$	389.15869	390.31	—	344, 252, 223, **151**, *107, 91*

[a] Compiled from peer-reviewed literature, and communications with USP collaborators. See corresponding chemical structures in *Figure 2*.
[b] **Bold**, fragment subjected to MS^3 fragmentation.
[c] *Italics*, MS^3 fragments derived from the parent fragment (**bold**).

Table 5. UV Absorbance Maxima and Retention Time Data for Select PDE5 Inhibitors[a]

#	Name	CAS Number	Chemical Formula	UV Absorbance Maxima (nm)	UV Absorbance Spectrum Type (*Figure 1*)	Retention Time (min)[b]	Relative Retention Time with Respect to Sildenafil
1	Acetaminotadalafil	1446144-71-3	$C_{23}H_{20}N_4O_5$	202, 222, 282	b	14.3	1.1
2	Acetil acid	147676-78-6	$C_{18}H_{20}N_4O_4$	230, 260, 285	—	—	—
3	Acetildenafil (Hongdenafil)	831217-01-7	$C_{25}H_{34}N_6O_3$	234, 282	e	12.5	1.0
4	Acetylvardenafil	1261351-28-3	$C_{25}H_{34}N_6O_3$	218, 246, 268(s)[c]	d	11.4	0.9
5	Aildenafil (Dimethylsildenafil, Methisosildenafil)	496835-35-9	$C_{23}H_{32}N_6O_4S$	226, 294	a	13.3	1.0
6	Aminotadalafil	385769-84-6	$C_{21}H_{18}N_4O_4$	200, 220, 284, 290(s)	b	14.2	1.1
7	(*S,R*)-Aminotadalafil [(+)-*trans*-Aminotadalafil]	1093940-70-5	$C_{21}H_{18}N_4O_4$	225, 283	—	—	—
8	Avanafil	330784-47-9	$C_{23}H_{26}N_7O_3Cl$	198, 244	f	13.0	1.0
9	Benzylsildenafil	1446089-82-2	$C_{28}H_{34}N_6O_4S$	291	—	—	—
10	*N*-Butylnortadalafil	171596-31-9	$C_{25}H_{25}N_3O_4$	222, 284	—	—	—
11	Carbodenafil (Fondenafil)	944241-52-5	$C_{24}H_{32}N_6O_3$	295	—	—	—
12	Chlorodenafil	1058653-74-9	$C_{19}H_{21}ClN_4O_3$	211, 235, 279	—	—	—
13	Chloropretadalafil	171489-59-1	$C_{22}H_{19}ClN_2O_5$	204, 222, 284	b	17.4	1.3
14	Cinnamyldenafil	1446089-83-3	$C_{32}H_{38}N_6O_3$	239	—	—	—
15	Cyclopentynafil	1173706-34-7	$C_{26}H_{36}N_6O_4S$	218, 290	—	—	—
16	Depiperazinothiosildenafil	1353018-10-6	$C_{17}H_{20}N_4O_4S_2$	295, 354	—	—	—

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Table 5. UV Absorbance Maxima and Retention Time Data for Select PDE5 Inhibitors[a] *(continued)*

#	Name	CAS Number	Chemical Formula	UV Absorbance Maxima (nm)	UV Absorbance Spectrum Type (*Figure 1*)	Retention Time (min)[b]	Relative Retention Time with Respect to Sildenafil
17	Descarbonsildenafil	1393816-99-3	$C_{21}H_{30}N_6O_4S$	225, 295	—	—	—
18	*N*-Desethylvardenafil	448184-46-1	$C_{21}H_{28}N_6O_4S$	226, 246(s)	d	11.9	0.9
19	Desmethylcarbodenafil	147676-79-7	$C_{23}H_{31}N_6O_3$	226,296	a	11.9	0.9
20	*N*-Desmethylsildenafil	139755-82-1	$C_{21}H_{28}N_6O_4S$	224, 294	a	12.9	1.0
21	Dimethylacetildenafil	1417999-76-8	$C_{25}H_{34}O_3N_6$	233, 276	—	—	—
22	Dioxo-acetildenafil	1609405-33-5	$C_{25}H_{30}N_6O_5$	No data	—	—	—
23	Dithio-desmethylcarbodenafil	1333233-46-7	$C_{23}H_{30}N_6OS_2$	258, 285, 356	—	—	—
24	Gendenafil	147676-66-2	$C_{19}H_{22}N_4O_3$	232, 274	f	16.8	1.3
25	Gisadenafil	334826-98-1	$C_{23}H_{33}N_7O_5S$	No data	—	—	—
26	Homosildenafil	642928-07-2	$C_{23}H_{32}N_6O_4S$	226, 292	a	13.3	1.0
27	Hydroxyacetildenafil (Hydroxyhongdenafil)	147676-56-0	$C_{25}H_{34}N_6O_4$	234, 280	e	12.2	0.9
28	Hydroxychlorodenafil	1391054-00-4	$C_{19}H_{23}ClN_4O_3$	212, 303	—	—	—
29	Hydroxyhomosildenafil	139755-85-4	$C_{23}H_{32}N_6O_5S$	226, 296	a	12.9	1.0
30	2-Hydroxypropylnortadalafil	1353020-85-5	$C_{24}H_{23}N_3O_5$	222, 284	—	—	—
31	Hydroxythiohomosildenafil (Hydroxyhomosildenafil thione, Sulfohydroxyhomosildenafil)	479073-82-0	$C_{23}H_{32}N_6O_4S_2$	228, 296, 352	c	15.0	1.2
32	Hydroxythiovardenafil	912576-30-8	$C_{23}H_{32}N_6O_4S_2$	203, 235, 316	—	—	—
33	Hydroxyvardenafil	224785-98-2	$C_{23}H_{32}N_6O_5S$	216	—	—	—
34	Imidazosagatriazinone (Desulfovardenafil)	139756-21-1	$C_{17}H_{20}N_4O_2$	212, 253	—	—	—
35	Isopiperazinonafil	1335201-06-3	$C_{25}H_{34}N_6O_4$	221, 290	—	—	—
36	Lodenafil carbonate	398507-55-6	$C_{47}H_{62}N_{12}O_{11}S_2$	226, 296	a	15.4	1.2
37	Mirodenafil	862189-95-5	$C_{26}H_{37}N_5O_5S$	216, 248	f	14.5	1.1
38	Mutaprodenafil	1387577-30-1	$C_{27}H_{35}N_9O_5S_2$	218, 240, 283, 297, 335	—	—	—
39	Nitrodenafil	147676-99-1	$C_{17}H_{19}N_5O_4$	212, 298	—	—	—
40	Nitroso-prodenafil	1266755-08-1	$C_{27}H_{35}N_9O_5S_2$	241, 301	—	—	—
41	Noracetildenafil (Demethylhongdenafil)	949091-38-7	$C_{24}H_{32}N_6O_3$	234, 280	e	12.3	0.9
42	Norneosildenafil (Piperidino sildenafil)	371959-09-0	$C_{22}H_{29}N_5O_4S$	226, 300	a	18.7	1.4
43	Norneovardenafil	358390-39-3	$C_{18}H_{20}N_4O_4$	215, 241	—	—	—
44	Nortadalafil (Demethyltadalafil)	171596-36-4	$C_{21}H_{17}N_3O_4$	No data	—	—	—
45	*N*-Octylnortadalafil (Octylnortadalafil)	1173706-35-8	$C_{29}H_{33}N_3O_4$	281	—	—	—
46	Oxohongdenafil	1446144-70-2	$C_{25}H_{32}N_6O_4$	481	—	—	—
47	Piperazinonafil (Piperazonifil, Dihydroacetildenafil)	1335201-04-1	$C_{25}H_{34}N_6O_4$	221, 290	—	—	—
48	Piperidino acetildenafil (Piperiacetildenafil)	147676-50-4	$C_{24}H_{31}N_5O_3$	234, 284	e	13.1	1.0
49	Piperidinovardenafil (Piperidenafil, Pseudovardenafil)	224788-34-5	$C_{22}H_{29}N_5O_4S$	224, 246(s)	d	16.5	1.3
50	Propoxyphenyl aildenafil	1391053-82-9	$C_{24}H_{34}N_6O_4S$	215, 225, 295	—	—	—

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Table 5. UV Absorbance Maxima and Retention Time Data for Select PDE5 Inhibitors[a] *(continued)*

#	Name	CAS Number	Chemical Formula	UV Absorbance Maxima (nm)	UV Absorbance Spectrum Type (*Figure 1*)	Retention Time (min)[b]	Relative Retention Time with Respect to Sildenafil
51	Propoxyphenyl hydroxyhomosildenafil (Methylhydroxyhomosildenafil)	139755-87-6	$C_{24}H_{34}N_6O_5S$	226, 294	a	13.4	1.0
52	Propoxyphenyl sildenafil	877777-10-1	$C_{23}H_{32}N_6O_4S$	226, 292	a	13.6	1.0
53	Propoxyphenyl thioaildenafil (Propoxyphenyl thiomethisosildenafil)	856190-49-3	$C_{24}H_{34}N_6O_3S_2$	227, 295, 355	—	—	—
54	Propoxyphenyl thiohydroxyhomosildenafil	479073-90-0	$C_{24}H_{34}N_6O_4S_2$	227, 295, 353	—	—	—
55	Sildenafil	139755-83-2	$C_{22}H_{30}N_6O_4S$	224, 294	a	13.0	1.0
56	Tadalafil (Tildenafil)	171596-29-5	$C_{22}H_{19}N_3O_4$	200, 222, 284, 292(s)	b	15.0	1.2
57	(–)-*trans*-Tadalafil (ent-Tadalafil)	629652-72-8	$C_{22}H_{19}N_3O_4$	231, 282, 289	—	—	—
58	Thioaildenafil (Sulfoaildenafil, Thiomethisosildenafil, Sulfodimethyl sildenafil, Dimethylthiosildenafil)	856190-47-1	$C_{23}H_{32}N_6O_3S_2$	228, 250(s), 296, 352, 366(s)	c	15.5	1.2
59	Thiohomosildenafil (Sulfohomosildenafil, Homosildenafil thione)	479073-80-8	$C_{23}H_{32}N_6O_3S_2$	228, 248(s), 296, 354, 370(s)	c	14.4	1.1
60	Thioquinapiperifil (KF31327)	220060-39-9	$C_{24}H_{28}N_6OS$	211, 268, 363	—	—	—
61	Thiosildenafil (Sulfosildenafil, Sildenafil thione)	479073-79-5	$C_{22}H_{30}N_6O_3S_2$	228, 250(s), 296, 356, 368(s)	c	15.2	1.2
62	Udenafil	268203-93-6	$C_{25}H_{36}N_6O_4S$	228, 298	f	13.6	1.0
63	Vardenafil	224785-90-4	$C_{23}H_{32}N_6O_4S$	226, 252(s)	d	12.1	0.9
64	Xanthoanthrafil (Benzamidenafil)	1020251-53-9	$C_{19}H_{23}N_3O_6$	202, 228, 278, 390	f	15.3	1.2

[a] Compiled from peer-reviewed literature, and contributed by the USP collaborators. See corresponding chemical structures in *Figure 2*.
[b] Retention times derived from the experiments conducted as described in *HPLC with Photodiode Array Detection* and in *HPLC with Mass-Spectrometric Detection* methods.
[c] (s) denotes a shoulder.

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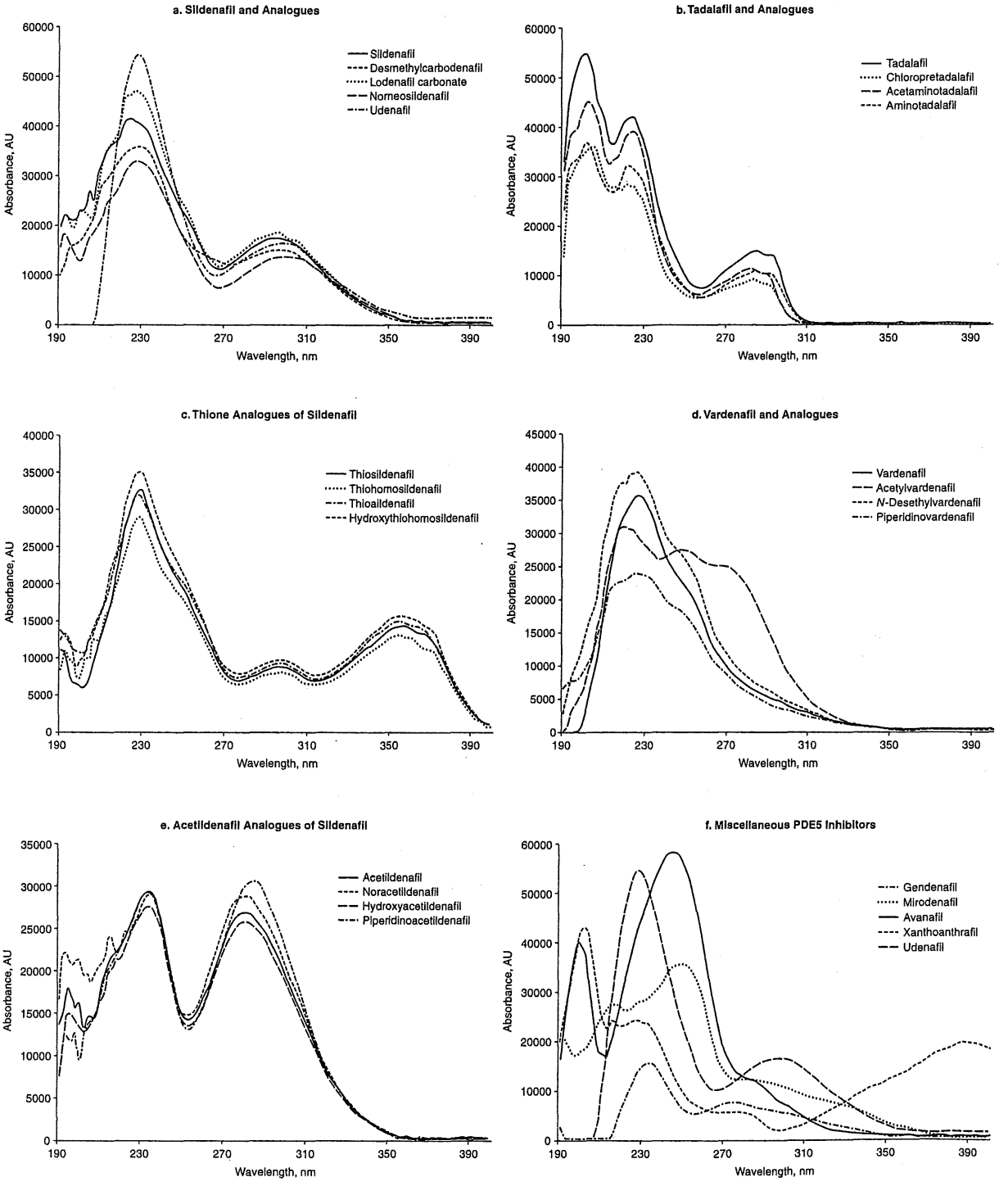

Figure 1. UV absorbance spectra of select PDE5 inhibitors.[11]

[11] Data contributed by USP collaborators.

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Figure 2. Chemical structures of select PDE5 inhibitors.[12]

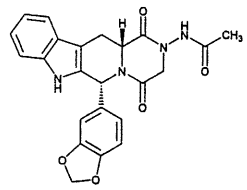
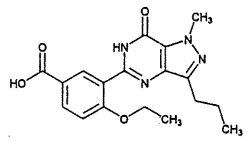
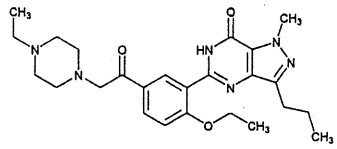
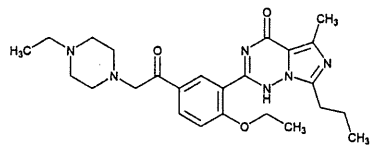
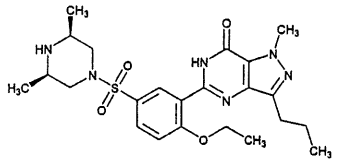
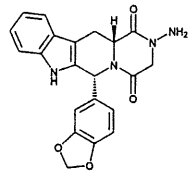
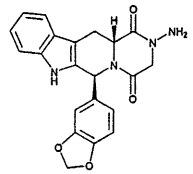
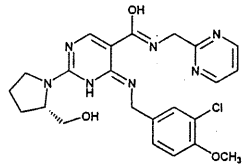
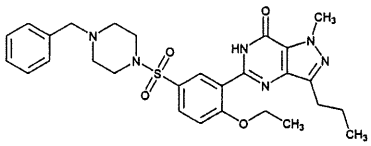
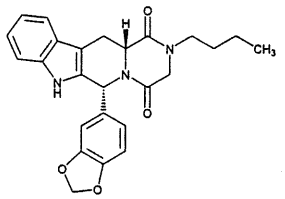
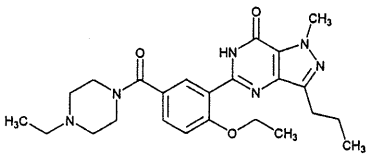
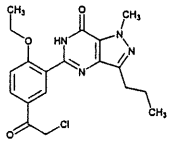
 Acetaminotadalafil	 Acetil acid
 Acetildenafil	 Acetylvardenafil
 Aildenafil	 Aminotadalafil
 (*S,R*)-Aminotadalafil	 Avanafil
 Benzylsildenafil	 *N*-Butylnortadalafil
 Carbodenafil	 Chlorodenafil

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Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*

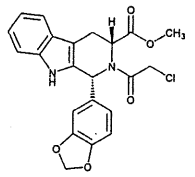
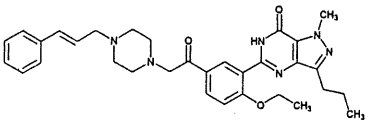
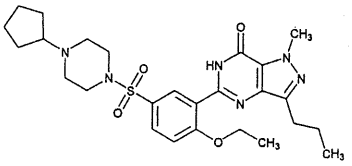
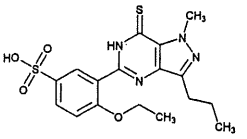
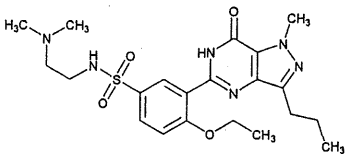
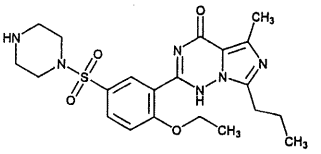
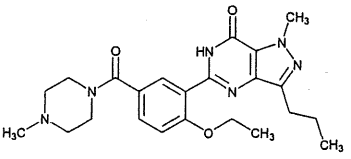
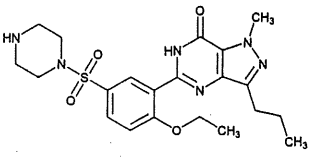
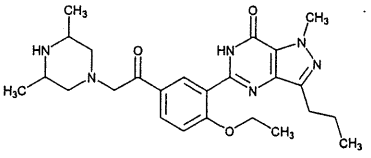
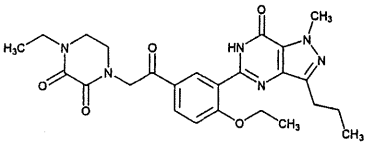
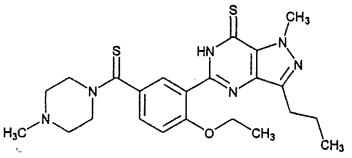
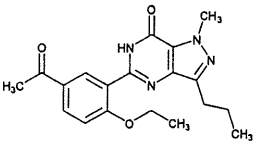
 Chloropretadalafil	 Cinnamyldenafil
 Cyclopentynafil	 Depiperazinothiosildenafil
 Descarbonsildenafil	 *N*-Desethylvardenafil
 Desmethylcarbodenafil	 *N*-Desmethylsildenafil
 Dimethylacetildenafil	 Dioxo-acetildenafil
 Dithio-desmethylcarbodenafil	 Gendenafil

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Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*

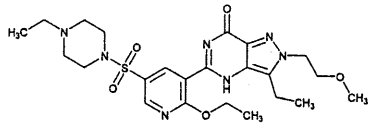
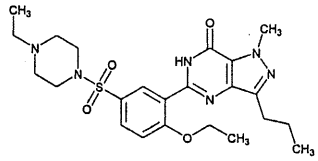
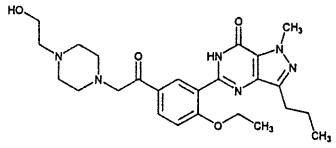
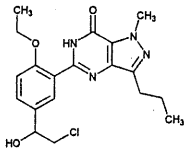
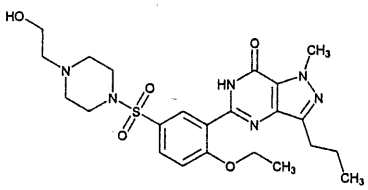
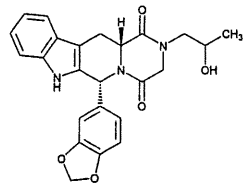
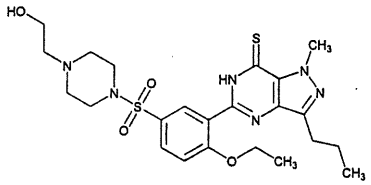
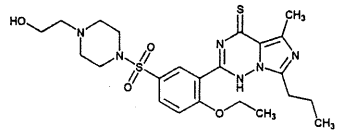
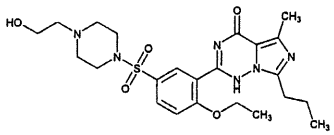
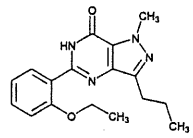
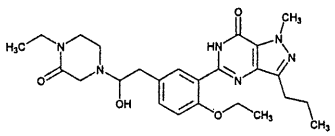
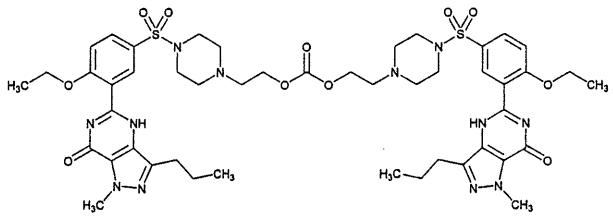
 Gisadenafil	 Homosildenafil
 Hydroxyacetildenafil	 Hydroxychlorodenafil
 Hydroxyhomosildenafil	 2-Hydroxypropylnortadalafil
 Hydroxythiohomosildenafil	 Hydroxythiovardenafil
 Hydroxyvardenafil	 Imidazosagatriazinone
 Isopiperazinonafil	 Lodenafil carbonate

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Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*

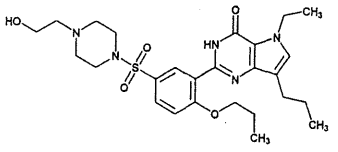
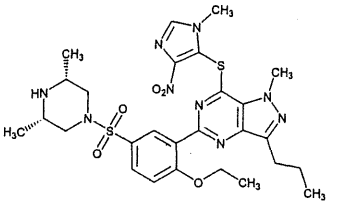
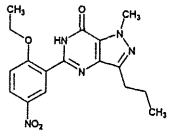
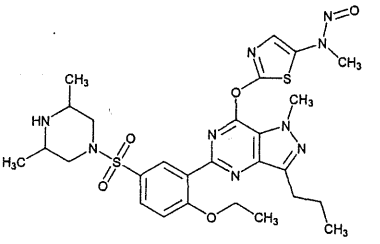
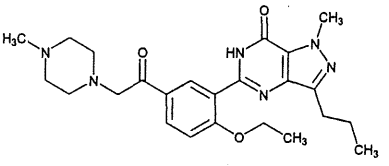
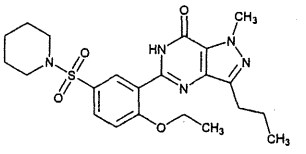
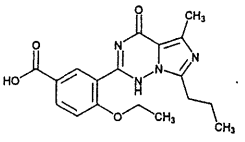
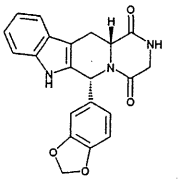
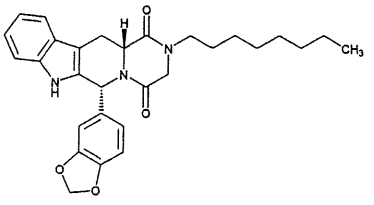
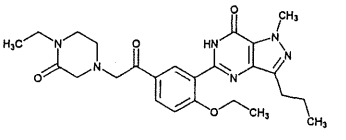
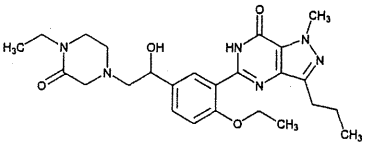
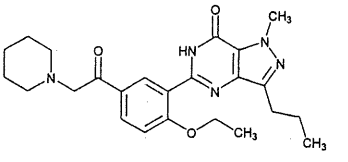
 Mirodenafil	 Mutaprodenafil
 Nitrodenafil	 Nitroso-prodenafil
 Noracetildenafil	 Norneosildenafil
 Norneovardenafil	 Nortadalafil
 *N*-Octylnortadalafil	 Oxohongdenafil
 Piperazinonafil	 Piperidino acetildenafil

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Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*

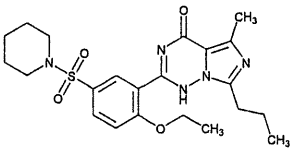
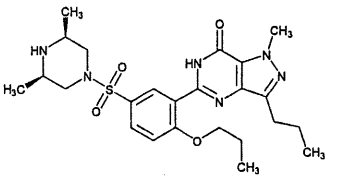
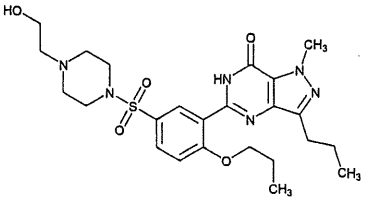
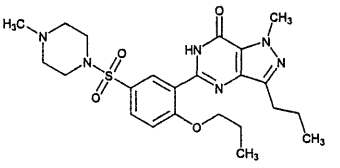
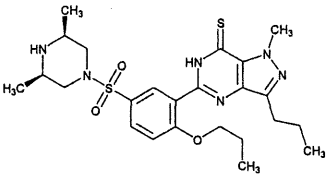
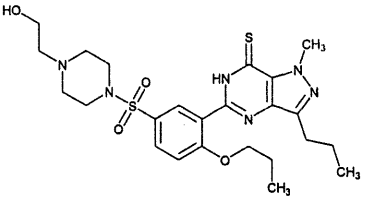
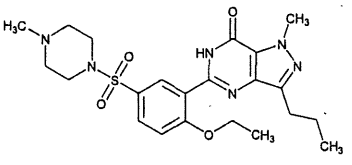
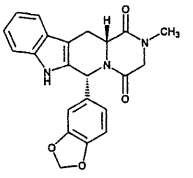
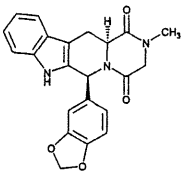
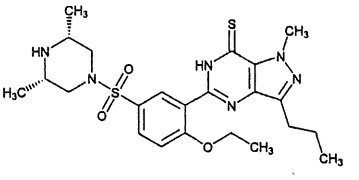
 Piperidinovardenafil	 Propoxyphenyl aildenafil
 Propoxyphenyl hydroxyhomosildenafil	 Propoxyphenyl sildenafil
 Propoxyphenyl thioaildenafil	 Propoxyphenyl thiohydroxyhomosildenafil
 Sildenafil	 Tadalafil
 (–)-*trans*-Tadalafil	 Thioaildenafil

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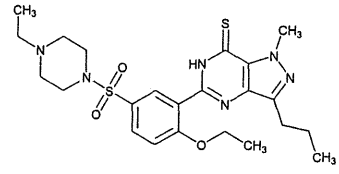
Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*



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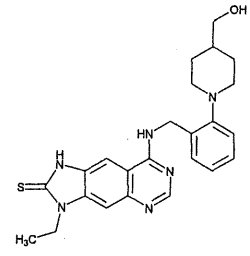

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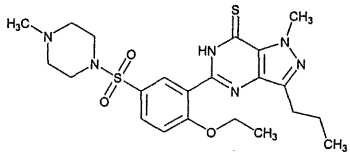

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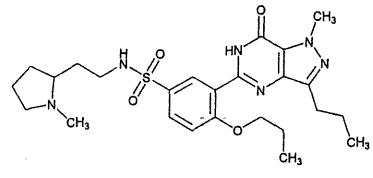

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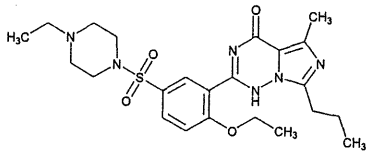

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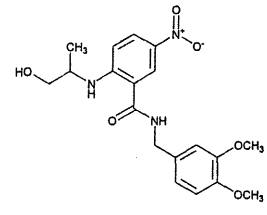

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⟨2750⟩ MANUFACTURING PRACTICES FOR DIETARY SUPPLEMENTS

GENERAL PROVISIONS

The principles included in this chapter contain recommended minimum current good manufacturing practices for the methods to be used in, and the facilities and controls to be used for, the manufacture, holding, packaging, labeling, and distribution of dietary ingredients and dietary supplements. These principles are set forth to ensure that such products meet the requirements of safety, have the identity and strength, and meet the quality and purity characteristics that they are represented to possess.

Excluded from this chapter are establishments engaged solely in the harvesting, storage, or distribution of one or more "raw agricultural commodities" as defined in Section 201(r) of the Federal Food, Drug, and Cosmetic Act [21 U.S.C. 321(r)], which are ordinarily cleaned, prepared, treated, or otherwise processed before being marketed to the consuming public.

The requirements pertaining to holding dietary ingredients and dietary supplements do not apply to holding those dietary supplements at a retail establishment for the sole purpose of direct retail sale to individual consumers. A retail establishment does not include a warehouse or other storage facility for a retailer or a warehouse or other storage facility that sells directly to individual consumers.

A *Glossary* of terms used in this chapter is presented at the end.

[12] Data compiled from published sources.

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ORGANIZATION AND PERSONNEL

Responsibilities of a Quality Control Unit

A quality control unit shall be established that has the responsibility and authority to approve or reject all raw materials, product containers, closures, in-process materials, packaging material, labeling, and finished dietary supplements, and the authority to review production records to ensure that no errors have occurred or, if errors have occurred, that they have been fully investigated. The quality control unit should be responsible for approving or rejecting products manufactured, processed, packed, or held under contract by another company.

Adequate laboratory facilities for the testing and approval (or rejection) of raw materials, product containers, closures, packaging materials, in-process materials, dietary ingredients, and dietary supplements should be available to the quality control unit.

The quality control unit should have the responsibility for approving or rejecting all procedures or specifications that impact on the identity, strength, quality, and purity of the dietary supplement. All responsibilities and procedures applicable to the quality control unit shall be in writing.

The designated person within the Quality Control Unit who conducts a material review and makes the disposition decision must, at the time of performance, document the material review and disposition decision made.

Personnel Qualifications

Each person engaged in the manufacture of dietary ingredients and dietary supplements should have the proper education, training, and experience (or any combination thereof) needed to perform the assigned functions. Training should be in the particular operation(s) that the employee performs as they relate to the employee's functions.

Appropriate documentation of training shall be retained by the company.

Each person responsible for supervising the manufacture of a dietary ingredient, a dietary supplement, or both should have the proper education, training, and experience (or any combination thereof) to perform assigned functions in such a manner as to provide assurance that the product has the safety, identity, strength, quality, and purity that it is represented to possess.

An adequate number of qualified personnel to perform and supervise the manufacture of each dietary ingredient, dietary supplement, or both products should be provided.

Personnel Responsibilities

The company management shall take all reasonable measures and precautions to ensure the following:

- **Disease control:** Any person who, by medical examination or supervisory observation, is shown to have, or appears to have: an illness; open lesion, including boils, sores, or infected wounds; or any other abnormal source of microbial contamination by which there is a reasonable possibility of an in-process or finished dietary ingredient or dietary supplement becoming adulterated; or processing equipment, utensils, or packaging materials becoming contaminated shall be excluded from any operations that may be expected to result in such adulteration or contamination until the condition is corrected. Personnel shall be instructed to report such health conditions to their supervisors.
- **Cleanliness:** All persons working in direct contact with raw materials, in-process or finished dietary ingredients and dietary supplements, processing equipment, utensils, or packaging materials shall conform to hygienic practices while on duty to the extent necessary to protect against adulteration or contamination of such materials. The methods for maintaining cleanliness include, but are not limited to, the following:
  - Wearing outer garments suitable to the operation in a manner that protects against the adulteration of raw materials or of in-process or finished dietary ingredients and dietary supplements, or contamination of processing equipment, utensils, or packaging materials;
  - Maintaining adequate personal cleanliness;
  - Removing cosmetics from parts of the body that may contact raw materials, in-process or finished dietary ingredients and dietary supplements, equipment, utensils, or containers;
  - Washing hands thoroughly (and sanitizing if necessary to protect against contamination with undesirable microorganisms) in an adequate hand-washing facility before starting work, after each absence from the work station, and at any other time when the hands may have become soiled or contaminated;
  - Removing all unsecured jewelry and other objects that might fall into raw materials, in-process or finished dietary ingredients and dietary supplements, equipment, or containers, and removing hand jewelry that cannot be adequately sanitized during periods in which in-process or finished product is manipulated by hand. If such hand jewelry and cosmetics cannot be removed, they may be covered by material that can be maintained in an intact, clean, and sanitary condition and that effectively protects against the adulteration of dietary ingredients and dietary supplements or contamination of processing equipment, utensils, or packaging materials;
  - Maintaining gloves, if they are used in raw materials or in in-process or finished product handling, in an intact, clean, and sanitary condition. The gloves should be of a material that adequately protects the product from contamination;
  - Wearing, where appropriate, in an effective manner, hair nets, caps, beard covers, or other effective hair restraints;
  - Storing clothing or other personal belongings in areas other than where in-process or finished product is exposed or where processing equipment or utensils are washed;
  - Confining the following actions to areas other than where in-process or finished product may be stored or exposed, or where processing equipment or utensils are washed: eating food, chewing gum, drinking beverages, or using tobacco; and

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- Taking any other necessary precautions to protect against adulteration of raw materials or of in-process or finished product; or contamination of processing equipment, utensils, or packaging materials with microorganisms or foreign substances, including but not limited to, perspiration, hair, cosmetics, tobacco, chemicals, and medicines applied to the skin.

GROUNDS, BUILDINGS, AND FACILITIES

Grounds

The grounds around a dietary ingredient manufacturing plant and a dietary supplement manufacturing plant under the control of the operator shall be kept in a condition that will protect against the adulteration of dietary ingredients and dietary supplements. The methods for adequate maintenance of grounds include, but are not limited to, the following:

- Properly storing equipment, removing litter and waste, and cutting weeds or grass within the immediate vicinity of the plant building or structures that may constitute an attractant, breeding place, or harborage for pests;
- Maintaining roads, yards, and parking lots so that they do not constitute a source of adulteration in areas where product is exposed;
- Adequately draining areas that may contribute to product adulteration by seepage, foot-borne filth, or providing a breeding place for pests; and
- Operating systems for waste treatment and disposal in an adequate manner so that they do not constitute a source of adulteration in areas where product is exposed. If the plant grounds are bordered by grounds not under the operator's control and not maintained in the manner described above, care shall be exercised in the plant by inspection, extermination, or other means to exclude pests, dirt, and filth that may be a source of product adulteration.

Building Design

Any building or buildings used in the manufacture of a dietary ingredient, a dietary supplement, or both should be of suitable size and shall be constructed in such a manner that floors, walls, and ceilings may be adequately cleaned and kept clean and in good repair; that drips or condensates from fixtures, ducts, and pipes do not adulterate raw materials or in-process or finished dietary ingredients and dietary supplements, or contaminate product containers, utensils, or packaging materials; and that aisles or working spaces are provided between equipment and walls and are adequately unobstructed and of adequate width to permit employees to perform their duties and to protect against adulterating in-process or finished product, or contaminating processing equipment with clothing or personal contact. Adequate screening or other protection against pests and insects should be installed, where necessary. The building should have adequate space for the orderly placement of equipment and materials to prevent mixups between different raw materials, product containers, closures, labeling, in-process materials, or finished products, and to prevent contamination. The flow of raw materials, product containers, closures, labeling, in-process materials, and products through the building or buildings should be designed to prevent contamination.

Operations should be performed within specifically defined areas of adequate size to prevent contamination or mixups or adulteration of in-process or finished dietary ingredients and dietary supplements, or contamination of processing equipment, utensils, or packaging materials with microorganisms, chemicals, filth, or other extraneous materials. The potential for mixups and product adulteration may be reduced by adequate product safety controls and operating practices or effective design, including the separation of operations in which contamination is likely to occur, by one or more of the following means: location, time, partition, airflow, enclosed systems, or other effective means. There should be separate or defined areas as follows:

- An area for the receipt, identification, storage, and withholding from use of components, product containers, closures, and labeling, pending the appropriate sampling, testing, or examination by the quality control unit before release for manufacturing or packaging;
- An area for the storage of released components, product containers, closures, and labeling;
- An area for storage of in-process materials;
- An area for manufacturing and processing operations;
- An area for packaging and labeling operations; and
- An area for control and laboratory operations.

Any building used in the manufacture of a dietary ingredient or a dietary supplement shall permit the taking of proper precautions to protect dietary ingredients or dietary supplements in outdoor bulk fermentation vessels by any effective means, including the following:

- Using protective coverings,
- Controlling areas over and around the vessels to eliminate harborages for pests,
- Checking on a regular basis for pests and pest infestation, and
- Skimming the fermentation vessels, as necessary.

Lighting

Adequate lighting shall be provided in all areas and should not expose bulk or finished product to adulteration or contamination. Adequate lighting should be provided in hand-washing areas, dressing and locker rooms, and toilet rooms, and in all areas where product is examined, processed, or stored and where equipment or utensils are cleaned; and such lighting should provide safety-type light bulbs, fixtures, skylights, or other glass suspended over exposed product in any step of preparation or otherwise protect against product adulteration in case of glass breakage.

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Ventilation, Air Filtration, Air Heating, and Cooling

Adequate ventilation shall be provided, as well as equipment for adequate control over microorganisms, dust, humidity, and temperature when used in the manufacture of a dietary ingredient and a dietary supplement to minimize odors and vapors (including steam and noxious fumes) in areas where they may adulterate dietary ingredients and dietary supplements; and to locate and operate fans and other air-blowing equipment in a manner that minimizes the potential for adulterating raw materials, in-process or finished dietary ingredients and dietary supplements, or contaminating processing equipment, utensils, or packaging materials.

Plumbing

The plumbing in the physical plant must be of an adequate size and design and be adequately installed and maintained to:

- Carry sufficient amounts of water to the required locations throughout the physical plant;
- Properly convey sewage and liquid disposable waste from the physical plant; and
- Avoid being a source of contamination to components, raw materials, dietary ingredients, dietary supplements, water supplies, or any contact surface, or creating an unsanitary condition.

Potable water at a suitable temperature, and under pressure as needed, should be supplied in a plumbing system free of defects that could contribute contamination to any dietary ingredients and dietary supplements. Potable water should meet the standards prescribed in the Environmental Protection Agency's Primary Drinking Water Regulations (40 CFR Part 141) or any state or local drinking water requirements that are more stringent. Water not meeting such standards should not be permitted in the potable water system for *Purified Water*. If potable water is to be used as a raw material, it should be further purified to satisfy compendial requirements.

Drains should be of adequate size and, where connected directly to a sewer, should have an air break or other mechanical device to prevent back-siphonage.

Sewage and Refuse

Sewage, trash, and other refuse in and from the building and immediate premises shall be disposed of in a safe and sanitary manner.

Washing and Toilet Facilities

Adequate washing facilities shall be provided, including hot and cold water, soap or detergent, air driers or single-service towels, and clean toilet facilities easily accessible to working areas.

General Maintenance and Sanitation

Any building used in the manufacture of a dietary ingredient, a dietary supplement, or both should be maintained in a clean and sanitary condition and shall be kept in repair sufficient to prevent raw materials and in-process or finished dietary ingredients and dietary supplements from becoming adulterated. It shall be free of infestation by rodents, birds, insects, and other vermin. Trash and organic waste matter shall be held and disposed of in a timely and sanitary manner.

Cleaning compounds and sanitizing agents used in cleaning and sanitizing procedures shall be free from undesirable microorganisms and shall be safe and adequate under the conditions of use. Compliance with this requirement may be verified by any effective means, including purchase of these substances under a supplier's guarantee or certification, or examination of these substances for contamination. Only the following toxic materials may be used or stored in a plant where product is processed or exposed:

- Those required to maintain clean and sanitary conditions,
- Those necessary for use in laboratory testing procedures,
- Those necessary for plant and equipment maintenance and operation, and
- Those necessary for use in the plant's operations.

Written procedures assigning responsibility for sanitation and describing in sufficient detail the cleaning schedules, methods, equipment, and materials to be used in cleaning the building and facilities shall be required.

Toxic cleaning compounds, sanitizing agents, and pesticide chemicals shall be identified, used, held, and stored in a manner that protects against adulteration of raw materials or of in-process or finished product, or contamination of processing equipment or packaging materials. All relevant regulations promulgated by other federal, state, and local government agencies for the application, use, or holding of these products should be followed.

No pests shall be allowed in any area of a dietary ingredient manufacturing plant and a dietary supplement manufacturing plant. Effective measures shall be taken to exclude pests from the processing areas and to protect against the adulteration by pests of product on the premises. The use of insecticides or rodenticides is permitted only under precautions and restrictions that will protect against the adulteration of raw materials, in-process or finished product, or contamination of processing equipment, utensils, or packaging materials.

Written procedures are also required for use of suitable rodenticides, insecticides, fungicides, fumigating agents, and cleaning and sanitizing agents. These procedures should be designed to prevent the contamination of equipment, raw materials, product containers, closures, packaging, labeling materials, or products. Rodenticides, insecticides, and fungicides should be registered and used in accordance with the Federal Insecticide, Fungicide, and Rodenticide Act.

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Sanitation procedures shall apply to work performed by contractors or temporary employees as well as work performed by full-time employees during the ordinary course of operations.

EQUIPMENT AND UTENSILS

Equipment and utensils used in the manufacture of dietary ingredients and dietary supplements shall be of appropriate design or selection, adequate size, and suitably located to facilitate operations for its intended use and for its cleaning and maintenance and to ensure that the specifications of dietary ingredients and dietary supplements are correct and are met.

Equipment and utensils include, but are not limited to, the following:

- Equipment used to hold or convey;
- Equipment used to measure;
- Equipment using compressed air or gas;
- Equipment used to carry out processes in closed pipes and vessels; and
- Equipment used in automatic, mechanical, or electronic systems.

Construction

All equipment and utensils shall be:

- Constructed so that surfaces that contact raw materials, in-process materials, or finished products are not reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the product beyond the established requirements;
- Made of nontoxic materials;
- Designed and constructed to withstand the environment in which they are used; the action of raw materials, in-process materials, dietary ingredients, or dietary supplements; and, if applicable, cleaning compounds and sanitizing agents; and
- Maintained to protect raw materials, in-process materials, dietary ingredients, and dietary supplements from being contaminated by any source.

Equipment and utensils must have seams that are smoothly bonded or maintained to minimize the accumulation of dirt, filth, organic material, particles of raw materials, in-process materials, dietary ingredients, or dietary supplements, or any other extraneous materials or contaminants, to minimize the opportunity for growth of microorganisms.

Each freezer, refrigerator, and cold storage compartment used to hold raw materials, in-process materials, dietary ingredients, or dietary supplements:

- Must be fitted with an indicating thermometer, temperature-measuring device, or temperature-recording device that shows, indicates, and records, or allows for recording by hand, the temperature accurately within the compartment; and
- Must have an automated device for regulating temperature or an automated alarm system to indicate a significant temperature change in a manual operation.

The design, construction, and use of equipment and utensils shall preclude the adulteration of raw materials, packaging materials, in-process materials, or finished product with any substances required for operation, such as:

- Lubricants,
- Fuel,
- Coolants,
- Metal or glass fragments,
- Filth or any other extraneous material,
- Contaminated water, or
- Any other contaminants.

Instruments or controls used in the manufacturing, packaging, labeling, or holding of a dietary ingredient, a dietary supplement, or both; and instruments or controls that are used to measure, regulate, or record temperatures, hydrogen-ion concentration (pH), water activity, or other conditions, and to control or prevent the growth of microorganisms or other contamination must be:

- Accurate and precise,
- Adequately maintained, and
- Adequate in number for their designated uses.

For any automated, mechanical, or electronic equipment that is used to manufacture, package, label, or hold a dietary ingredient, a dietary supplement, or both:

- The suitability of the equipment must be determined by ensuring that the equipment is capable of operating satisfactorily within the operating limits required by the process;
- The equipment must be routinely calibrated, inspected, or checked to ensure proper performance. The quality control unit must approve these calibrations, inspections, or checks;
- The appropriate controls for automated, mechanical, and electronic equipment (including software for a computer-controlled process) must be established and used to ensure that any changes to the manufacturing, packaging, labeling, holding, or other operations are approved by the quality control unit and instituted only by authorized personnel; and
- The appropriate controls must be established and used to ensure that the equipment functions in accordance with its intended use. These controls must be approved by the quality control unit.

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Compressed air or other gases introduced mechanically into or onto raw materials, in-process materials, dietary ingredients, dietary supplements, or contact surfaces, or that are used to clean any contact surface, must be treated in such a way that the raw material, in-process material, dietary ingredient, dietary supplement, or contact surface is not contaminated.

Cleaning and Maintenance

Equipment and utensils shall be cleaned, maintained, and sanitized at adequate intervals, between the manufacture of different batches of the same product and between the manufacture of different products, to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the product beyond the established requirements.

In wet processing during manufacturing, all contact surfaces must be cleaned and sanitized, as necessary, to protect against the introduction of microorganisms into components, dietary ingredients, or dietary supplements. When cleaning and sanitizing are necessary, all contact surfaces must be cleaned and sanitized before use and after any interruption during which the contact surface may have become contaminated.

In a continuous production operation or in back-to-back consecutive operations, which involve different batches of the same dietary ingredient or dietary supplement, the contact surfaces must be adequately cleaned and sanitized.

The surfaces that do not come into direct contact with raw materials, in-process materials, dietary ingredients, or dietary supplements must be cleaned as frequently as necessary to protect against contaminating raw materials, in-process materials, dietary ingredients, or dietary supplements.

Single-service articles (such as utensils intended for one-time use, paper cups, and paper towels) must be:

- Stored in appropriate containers; and
- Handled, dispensed, used, and disposed of in a manner that protects against contamination of raw materials, in-process materials, dietary ingredients, dietary supplements, or any contact surface.

Cleaning compounds and sanitizing agents must be adequate for their intended use and safe under their conditions of use.

The portable equipment and utensils that have contact surfaces must be cleaned, sanitized, and then stored in a location and manner that protects them from contamination.

Written procedures for cleaning and maintaining equipment, including utensils, used in the manufacture of a product should be established and followed. These procedures should include, but are not necessarily limited to, the following:

- Assignment of responsibility for cleaning and maintaining equipment;
- Maintenance and cleaning schedules, including, where adequate, sanitizing schedules;
- A description in sufficient detail of the methods, equipment, and materials used in cleaning and maintenance operations, and the methods of disassembling and reassembling equipment, as necessary, to ensure proper cleaning and maintenance;
- Removal or obliteration of previous batch identification;
- Identification and protection of clean equipment from contamination before use;
- Inspection of equipment for cleanliness immediately before use;
- Regular calibration and inspection of equipment, or checking machines, to ensure proper performance and function must be conducted:
  - Before first use, and
  - At a frequency specified in writing by supporting references.
- Instruments or controls that cannot be adjusted to agree with the reference standard must be repaired or replaced.

A written record of calibration, inspection, maintenance of equipment, and major equipment cleaning and use shall be maintained in individual equipment logs that show the date, product, and lot number of each batch processed. The persons performing the cleaning shall record in the log that the work was performed. Entries in the log should be in chronological order.

The following is specified to keep records related to automated or electric equipment:

- There must be backup file(s) of current software programs (and of outdated software that is necessary to retrieve records that are required to be retained, in accordance with the section *Records and Reports* in this chapter, when current software is not able to retrieve such records) and of data entered into computer systems used to manufacture, package, label, or hold dietary supplements.
  - A backup file (e.g., a hard copy of data entered, diskettes, tapes, microfilm, or compact disks) must be an exact and complete record of the data entered.
  - Backup software programs and data must be kept secure from alterations, inadvertent erasures, or loss.

RAW MATERIALS, PRODUCT CONTAINERS, AND CLOSURES

Written procedures describing in sufficient detail the receipt, identification, storage, handling, sampling, testing, and approval or rejection of raw materials, product containers, and closures should be provided.

Raw materials, product containers, and closures at all times should be handled and stored in a manner to prevent contamination.

Raw agricultural materials that contain soil or other contaminants shall be washed or cleaned as necessary. Water used for washing, rinsing, or conveying raw agricultural materials shall be safe and of adequate sanitary quality. Notwithstanding the general requirement for potable water, water may be reused for washing, rinsing, or conveying raw agricultural materials if it does not increase the level of contamination of such materials.

Bagged or boxed raw materials of product containers or closures should be stored off the floor and suitably spaced to permit cleaning and inspection.

Each lot should be appropriately identified as to its status (i.e., quarantined, approved, or rejected).

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Receipt and Storage of Untested Raw Materials, Product Containers, and Closures

Written procedures shall be established and followed describing the receipt, identification, examination, handling, and sampling of raw materials. Upon receipt and before acceptance, each container or grouping of containers of raw materials, product containers, and closures should be examined visually for appropriate labeling as to contents, container damage, or broken seals, and for contamination. They are then stored under quarantine until they have been tested or examined, as appropriate, and released.

Raw materials shall be held in bulk, or in containers designed and constructed so as to protect against adulteration, and shall be held at such temperature and relative humidity and in such a manner as to prevent a dietary ingredient or dietary supplement from becoming adulterated. Frozen raw materials and other ingredients shall be kept frozen. If thawing is required before use, it shall be done in a manner that prevents the raw materials and other ingredients from becoming adulterated within the meaning of the Act.

Testing and Approval or Rejection

Each lot of raw materials, product containers, and closures should be sampled, tested, or examined, as appropriate, and released for use by the quality control unit. On the basis of adequate process verification, in-process controls, and statistical confidence, a skip-lot testing plan is an alternative to fully testing every batch provided that at least one identity test is conducted. An appropriate amount of each lot of raw materials should be reserved for 1 year beyond the shelf life appearing on the label of finished dietary supplements in which the raw materials were used. If adverse event reports are received (see the subsection *Adverse Event Reports*), the reserved raw materials should be kept for 6 years (serious events) or 3 years (nonserious events) from the date the first report is received.

Representative samples should be collected for testing or examination. Sampling of botanicals should be in compliance with the provisions set in *Articles of Botanical Origin* ⟨561⟩. The number of containers sampled, and the amount of material taken from each container, should be based upon appropriate criteria such as statistical criteria for raw material variability, confidence levels, and degree of precision desired, the past quality history of the supplier, and the quantity needed for analysis and reserve where required. The following procedures should be used to collect the samples:

- The containers of raw materials selected should be cleaned, where necessary, by adequate means.
- The containers should be opened, sampled, and resealed in a manner designed to prevent contamination of their contents and contamination of other raw materials, product containers, or closures.
- These containers should be identified so that the following information can be determined: name of the material sampled, the lot number, the container from which the sample was taken, the date on which the sample was taken, and the name of the person who collected the sample.

Use the following procedure to examine and test the samples:

- At least one test should be conducted to verify the identity of each raw material of a product even in cases where skip-lot testing is used. Such tests may include any appropriate test with established sufficient specificity to determine identity, including chemical and laboratory tests, gross organoleptic analysis, microscopic identification, or analysis of constituent markers.
- Each raw material should be tested for conformity with all appropriate written specifications for purity, strength, and quality. However, a report of analysis may be accepted from the supplier of a raw material, provided that the manufacturer establishes the reliability of the supplier's analyses and provided that at least one identity test is conducted on such raw material by the manufacturer.
- Containers and closures should be tested for conformance with all appropriate written procedures. However, a certificate of testing may be accepted from the supplier, provided that at least a visual identification is conducted on such containers or closures by the manufacturer.
- Each lot of a raw material, rework, product container, or closure that is liable to contamination with filth, insect infestation, or other extraneous adulterant should be examined against established specifications for such contamination and shall comply with any applicable Food and Drug Administration regulations and guidelines. Skip-lot examination should not apply in such cases.
- Each lot of a raw material that is liable to microbiological contamination that is objectionable in view of its intended use shall be subjected to microbiological tests before use. Raw materials either shall not contain levels of microorganisms that may produce food poisoning or other disease in humans, or shall be otherwise treated during manufacturing operations so that they no longer contain levels that would cause the product to be adulterated within the meaning of the Act. In lieu of such testing by the manufacturer, a guarantee or certification of analysis may be accepted from the supplier of a component provided that the manufacturer establishes the reliability of the supplier's analysis.
- Raw materials and other ingredients susceptible to adulteration with aflatoxin, other natural toxins, pesticides, or heavy metals shall comply with current Food and Drug Administration regulations, guidelines, and action levels for poisonous or deleterious substances and the requirements in ⟨561⟩, or in each monograph, before these materials or ingredients are incorporated into a finished dietary ingredient or dietary supplement. Compliance with this requirement may be accomplished by analyzing these materials and ingredients for aflatoxins and other natural toxins; or, in lieu of such testing by the manufacturer, a guarantee or certification of analysis may be accepted from the supplier of a raw material provided that the manufacturer establishes the reliability of the supplier's analysis.
- Any lot of raw material, product container, or closure that meets the appropriate written specifications of identity, strength, quality, and purity and related tests may be approved and released for use. Any lot of such material that does not meet such specifications should be rejected.

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Use of Approved Raw Materials, Product Containers, and Closures

Raw materials, product containers, and closures approved for use should be rotated so that the oldest approved stock is used first. Deviation from the requirement is permitted if such deviation is temporary and adequate.

Retesting of Approved Raw Materials, Product Containers, and Closures

Raw materials, product containers, and closures should be retested or reexamined, as appropriate, for identity, strength, quality, and purity and approved or rejected by the quality control unit after a specified time in storage or as necessary, e.g., after exposure to air, heat, or other conditions that might adversely affect the raw material, product container, or closure or after storage of active and inactive ingredients and in-process materials for long periods of time.

Rejected Raw Materials, Product Containers, and Closures

Rejected raw materials, product containers, and closures should be identified and controlled under a quarantine system that prevents their use in manufacturing or processing operations for which they are unsuitable.

PRODUCTION AND PROCESS CONTROLS

Written Procedures

Written procedures should be provided for production and process controls designed to ensure that the dietary ingredients and dietary supplements have the identity, strength, quality, and purity that they are represented to possess. These procedures should be drafted, reviewed, and approved by the appropriate organizational units and reviewed and approved by the quality control unit. These production and process control procedures should be followed in the execution of the various production and process control functions and should be documented at the time of performance. Any deviation from the written procedures should be recorded and justified.

- All operations in the receiving, inspecting, transporting, segregating, preparing, manufacturing, packaging, and storing of dietary ingredients and dietary supplements shall be conducted in accordance with adequate sanitation principles.
- All reasonable precautions shall be taken to ensure that production procedures do not contribute adulteration from any source. Chemical, microbial, or extraneous-material testing procedures shall be used where necessary to identify sanitation failures or possible product adulteration.
- All product that has become contaminated to the extent that it is adulterated within the meaning of the Act shall be rejected, or if permissible, treated or processed to eliminate the contamination.
- All product manufacturing, including packaging and storage, shall be conducted under such conditions and controls as are necessary to minimize the potential for the growth of microorganisms, or for the adulteration of raw materials, in-process materials, and finished product.
- Measures taken to destroy microorganisms, reduce the microbial load, or prevent the growth of undesirable microorganisms, particularly those of public health significance, shall be adequate under the conditions of manufacture, handling, and distribution to prevent dietary supplements and ingredients from being adulterated within the meaning of the Act. These measures shall also comply with current regulations affecting dietary supplement products and ingredients.
- Work-in-process shall be handled in a manner that protects against adulteration.
- In-process material must be held under appropriate conditions of temperature, humidity, and light.
- Effective measures shall be taken to protect finished dietary ingredients and dietary supplements from adulteration by raw materials, in-process materials, or refuse. When raw materials, in-process materials, or refuse are unprotected, they shall not be handled simultaneously in a receiving, loading, or shipping area if that handling could result in adulterated dietary ingredients and dietary supplements. Dietary ingredients and dietary supplements transported by conveyor shall be protected against adulteration as necessary.
- Effective measures shall be taken as necessary to protect against the inclusion of metal or other extraneous material in product. Compliance with this requirement may be accomplished by using sieves, traps, magnets, electronic metal detectors, or other suitable effective means.
- Mechanical manufacturing steps such as cutting, sorting, inspecting, shredding, drying, grinding, blending, and sifting shall be performed so as to protect dietary ingredients and dietary supplements against adulteration. Compliance with this requirement may be accomplished by providing adequate physical protection of dietary ingredients and dietary supplements from contact with adulterants. Protection may be provided by adequate cleaning and sanitizing of all processing equipment between each manufacturing step.
- Heat blanching, when required in the preparation of a dietary ingredient or a dietary supplement, should be effected by heating the product to the required temperature, holding it at this temperature for the required time, and then either rapidly cooling the material or passing it to subsequent manufacturing without delay. Thermophilic growth and contamination in blanchers should be minimized by the use of adequate operating temperatures and by periodic cleaning. Where the blanched product is washed before filling, potable water shall be used.
- Intermediate of dehydrated dietary ingredients and dietary supplements that rely on the control of water (a_w) for preventing the growth of undesirable microorganisms shall be processed to and maintained at a safe moisture level. Compliance with this requirement may be accomplished by any effective means, including employment of one or more of the following practices:

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- Monitoring the water activity (a_w) of the material;
- Controlling the soluble solids–water ratio in finished product; and
- Protecting finished product from moisture pickup, by use of a moisture barrier or by other means, so that the water activity (a_w) of the product does not increase to an unsafe level.

- Dietary ingredients and dietary supplements that rely principally on the control of pH for preventing the growth of undesirable microorganisms shall be monitored and maintained at an appropriate pH. Compliance with this requirement may be accomplished by any effective means, including employment of one or more of the following practices:
  - Monitoring the pH and water activity, if appropriate, of raw materials, in-process material, and finished product; and
  - Controlling the amount of acid added to the product.
- When ice is used in contact with dietary ingredients and dietary supplements, it shall be made from potable water, and shall be used only if it has been manufactured in accordance with current good manufacturing practice in manufacturing, packing, or holding human food as outlined in 21 CFR Part 110.

Charge-In of Raw Materials

Written production and control procedures should include the following, which are designed to ensure that the dietary supplements have the identity, strength, quality, and purity that they are represented to possess:

- The batch should be formulated with the intent to provide NLT 100% of the labeled or established amount of dietary ingredient.
- Raw materials for product manufacturing should be weighed, measured, or subdivided as appropriate and the appropriate signatures recorded in the batch record.
- Actual yields and percentages of theoretical yield should be determined at appropriate phases of processing.

Material scheduled for rework shall be identified as such.

Equipment Identification

All compounding and storage containers, processing lines, and major equipment used during the production of a batch of a product should be properly identified to indicate their contents and, when necessary, the phase of processing of the batch.

Sampling and Testing of In-Process Materials, Dietary Ingredients, and Dietary Supplements

To ensure batch uniformity and integrity of dietary supplements, written procedures should be established and followed that describe the in-process controls and tests or examinations to be conducted on appropriate samples of in-process materials. On the basis of process verification, in-process controls, and statistical confidence, a skip-lot testing plan is an alternative to testing every batch of finished products provided that at least one representative measure is performed. Control procedures should be established to monitor the output of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the finished product. Such control procedures may include, but are not limited to, the following, where appropriate:

- Friability
- Weight variation
- Disintegration time
- Dissolution time
- Clarity, completeness, or pH of solutions
- Blend uniformity

In-process specifications for such characteristics should be consistent with finished product specifications. Examination and testing of samples should ensure that the in-process material and dietary supplement conform to the established specifications.

In-process materials should be tested for identity, strength, quality, and purity as adequate, and approved or rejected by the quality control unit during the production process, e.g., at commencement or completion of significant phases or after storage for long periods.

Rejected or adulterated in-process materials should be identified and controlled under a quarantine system designed to prevent their use in manufacturing or processing operations for which they are unsuitable and to prevent the adulteration of other products.

LABELING AND PACKAGING

Materials Examination and Usage Criteria

Written procedures should be provided describing in sufficient detail the receipt, identification, storage, handling, sampling, examination, testing of labeling and packaging materials, or products received for packaging or labeling. Each immediate container or grouping of immediate containers in a shipment of product received for packaging or labeling, or of packaging and labeling materials, must be visually examined for appropriate content label, container damage, or broken seals to determine whether the container condition may have resulted in contamination or deterioration of the received product. The supplier's invoice, guarantee, or certification in a shipment of the received product must be visually examined to ensure that the received

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product is consistent with the purchase order. Labeling and packaging materials or products received for packaging or labeling should be quarantined until:

- Representative samples of each unique shipment, and of each unique lot within each unique shipment, of received product for packaging or labeling, or of packaging and labeling materials, are collected;
- The quality control unit reviews and approves the documentation to determine whether the received product for packaging or labeling, or packaging and labeling materials, meets the specifications; and
- The quality control unit approves the received product for packaging or labeling, or packaging and labeling materials, and releases for use from quarantine.

Those that do not meet such specifications should be identified and rejected to prevent their use in operations for which they are unsuitable.

A record should be kept of each shipment received of each different labeling and packaging material, or each different received product for packaging or labeling, indicating receipt, date of examination or testing, and whether accepted or rejected.

Each unique lot within each unique shipment of received product for packaging or labeling, or of packaging and labeling materials, must be identified in a manner that allows the recipient to trace the lot to the supplier, the date received, the name of the received product, the status of the received product (e.g., quarantined, approved, or rejected), and to the product that was packaged or labeled and distributed.

This unique identifier must be used whenever the disposition of each unique lot within each unique shipment of the received product for packaging or labeling, or of packaging and labeling materials, is recorded.

Labels and other labeling materials for each different product, strength, product type, or quantity of contents should be stored separately under conditions that will protect against contamination and deterioration and avoid mixups. Only authorized personnel should have access to the storage area.

Packaging and labels must be held under appropriate conditions so that the packaging and labels are not adversely affected (e.g., contamination or deterioration).

Gang printing of labeling to be used for different products or different strengths of the same product (or labeling of the same size and identical or similar format or color schemes) should be minimized. If gang printing is employed, packaging and labeling operations should provide for special control procedures, taking into consideration sheet layout, stacking, cutting, and handling during and after printing.

Printing devices on, or associated with, manufacturing lines used to imprint labeling upon the product unit label or case should be monitored to ensure that all imprinting conforms to the print specified in the batch production record.

Obsolete and outdated labels, labeling, other packaging materials, and products received for packaging or labeling should be destroyed and documented.

Labeling Issuance

Strict control should be exercised over labeling issued for use in product labeling operations. The control procedures employed should be in writing with sufficient detail.

Labeling materials issued for a batch should be carefully examined for identity and conformity to the labeling specified in the master and batch production records.

Procedures should be used to reconcile the quantities of labeling issued, used, and returned, and should require evaluation of discrepancies found. If discrepancies are found between the quantity of product finished and the quantity of labeling issued and are outside preset limits based on historical operating data, such discrepancies should be investigated.

Returned labeling should be maintained and sorted in a manner to prevent mixups and provide proper identification.

All excess labeling bearing lot or control numbers should be destroyed and documented.

Operations

Written procedures designed to ensure that correct labels, labeling, and packaging materials are used for dietary supplements should incorporate the following features:

- Prevention of mixups and cross-contamination by physical or spatial separation from operations on other products;
- Identification of the product with a lot or control number;
- Examination of packaging and labeling materials for suitability and correctness before packaging operations, and documentation of such examination in the batch production record; and
- Inspection of the packaging and labeling facilities immediately before use to ensure that all products have been removed from previous operations. Inspection should also be made to ensure that packaging and labeling materials not suitable for subsequent operations have been removed. Results of the inspection should be documented in the batch production records.

Relabeling and Repackaging

- Dietary ingredients and dietary supplements may be repackaged or relabeled only after the quality control unit has approved such repackaging or relabeling.
- A representative sample of each batch of repackaged or relabeled dietary ingredients and dietary supplements must be examined to determine whether the repackaged or relabeled dietary ingredients and dietary supplements meet all established specifications.
- The quality control unit must approve or reject each batch of repackaged or relabeled dietary ingredients and dietary supplements before its release for distribution.

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Tamper-Resistant Packaging

REQUIREMENTS

Each manufacturer and packer who packages a dietary supplement for retail sale shall package the product in a tamper-resistant package, if this product is accessible to the public while held for sale. A tamper-resistant package is one having an indicator or barrier to entry which, if breached or missing, can reasonably be expected to provide visible evidence to consumers that tampering has occurred. To reduce the likelihood of substitution of a tamper-resistant feature after tampering, the indicator or barrier to entry is required to be distinctive by design or by the use of an identifying characteristic (e.g., a pattern, name, registered trademark, logo, or picture). For purposes of this section, the term "distinctive by design" means that the packaging cannot be duplicated with commonly available materials or through commonly available processes. A tamper-resistant package may involve an immediate-container and closure system, or secondary-container or carton system, or any combination of systems intended to provide a visual indication of package integrity. The tamper-resistant feature should be designed to remain intact when handled in a reasonable manner during manufacture, distribution, and retail display.

LABELING

Each retail package of a dietary supplement covered by this section shall bear a statement that is prominently placed so that consumers are alerted to the specific tamper-resistant feature of the package. The labeling statement should be so placed that it will be unaffected if the tamper-resistant feature of the packaging is breached or missing. If the tamper-resistant feature chosen to meet the requirement above is one that uses an identifying characteristic, that characteristic should be referred to in the labeling statement. For example, the labeling statement on a bottle with a shrink band could say "For your protection, this bottle has an imprinted seal around the neck."

Dietary Supplement Examination

Packaged and labeled products should be examined during finishing operations to ensure that containers and packages in the lot have the correct label. A representative sample of units should be collected at the completion of finishing operations and visually examined for correct labeling. Results of these examinations should be recorded in the batch production or control records.

Contact Information

The manufacturer, packer, or distributor of dietary supplements is required to comply with the current labeling requirements in the law that also include a domestic address or phone number through which an adverse event report for a dietary supplement may be received.

Shelf Life

Dietary supplements should bear a date indicative of their shelf life, determined by appropriate testing, to ensure that they meet applicable standards of identity, strength, quality, and purity at or before the labeled shelf-life date.

Shelf life should be related to any storage conditions stated on the labeling.

HOLDING AND DISTRIBUTION

Warehousing Procedures

Storage and transportation of the finished product shall be under conditions that will protect the product against physical, chemical, and microbial adulteration as well as against deterioration of the product and the container.

Written procedures describing the warehousing of dietary supplements should be established and followed and should include the following:

- Quarantine of finished products before disposition by the quality control unit; and
- Storage of finished products under appropriate conditions of temperature, humidity, and light so that the identity, strength, quality, and purity of the products are not affected.

Distribution Procedures

Written procedures describing the distribution of dietary supplements shall be established and followed and should include the following:

- A procedure whereby the oldest approved stock of a product is distributed first. (Deviation from this requirement is permitted if such deviation is temporary and adequate.)
- A system by which the distribution of each lot of product can be readily determined to facilitate its recall if necessary.

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QUALITY CONTROL OPERATIONS

The establishment of any specifications, standards, sampling plans, test procedures, or other laboratory control mechanisms required by this chapter, including any change in such specifications, standards, sampling plans, test procedures, or other laboratory control mechanisms, shall be drafted by the appropriate organizational unit and reviewed and approved by the quality control unit. The requirements in this section should be followed and documented at the time of performance. Any deviation from the written specifications, standards, sampling plans, test procedures, or other laboratory control mechanisms shall be recorded and justified.

Quality control operations include the establishment of scientifically sound and appropriate specifications, standards, sampling plans, and test procedures designed to ensure that raw materials, product containers, closures, in-process materials, labeling, products received for labeling and packaging operations as dietary supplements, and finished products conform to adequate standards of identity, strength, quality, and purity. These controls include the following:

- Determination of conformance to appropriate written specifications for the acceptance of each lot within each shipment of raw materials, product containers, closures, and labeling used in the manufacture of dietary ingredients and dietary supplements, and of products received for labeling and packaging operations as dietary supplements. (The specifications include a description of the sampling and testing procedures used. Samples should be representative and adequately identified. Such procedures also require appropriate retesting of any raw material, product container, or closure that is subject to deterioration.) On the basis of adequate process verification, in-process controls, and statistical confidence, a skip-lot testing plan is an alternative to testing every batch, excluding raw materials, which require 100% identity testing.
- Determination of conformance to written specifications and a description of sampling and testing procedures for in-process materials. (Such samples should be representative and properly identified.)
- Determination of conformance to written descriptions of sampling procedures and appropriate specifications for finished products. (Such samples should be representative and properly identified.)
- The calibration of instruments, at suitable intervals, in accordance with an established written program containing specific directions, schedules, limits for accuracy and precision, and provisions for remedial action in the event that accuracy and/or precision limits are not met. Instruments not meeting established specifications shall not be used until repaired.

Testing and Release for Distribution

There should be appropriate laboratory determination of satisfactory conformance to specifications for the finished product, including the identity and strength before release. On the basis of adequate process verification, in-process controls, or statistical confidence, a skip-lot or composite testing plan is an alternative to testing every batch.

There should be appropriate laboratory testing, as necessary, of each batch of dietary supplement required to be free of objectionable microorganisms. The accuracy, linearity, sensitivity, specificity, and precision of test methods employed by the firm, when they differ from compendial methods, should be established and documented.

Written procedures should describe any sampling and testing plans, which should include the method of sampling and the number of units per batch to be tested.

Products failing to meet established standards or specifications and any other relevant quality control criteria should be rejected. Rejected or adulterated dietary ingredients and dietary supplements shall be identified, stored, and disposed of in a manner that protects against the adulteration of the other products. Reprocessing may be performed. Before acceptance and use, reprocessed material must meet established standards, specifications, and any other relevant criteria. Written procedures shall be established and followed prescribing the method for reprocessing batches or operations start-up materials that do not conform to finished goods standards or specifications. Finished goods manufactured using such materials shall meet all established purity, composition, and quality standards.

Stability Testing

There should be a written protocol designed to assess the stability characteristics of dietary supplements. The results of such testing should be used in determining appropriate storage conditions and shelf life. This protocol should include the following:

- Sample size and test intervals based on statistical criteria for each attribute should be examined to ensure valid estimates of stability;
- Storage conditions for samples retained for testing;
- Reliable, meaningful, and specific test methods should be used; and
- The dietary supplement should be tested in the same type of container–closure system as that in which the dietary supplement is marketed.

An adequate number of batches of each dietary supplement should be tested to determine an adequate shelf life, and a record of these data should be maintained. Accelerated studies combined with basic stability information on the raw materials, dietary supplements, and container–closure systems may be used to support tentative shelf life if full shelf-life studies are not available. Simplified stability testing procedures may be used where data from similar product formulations are available to support a shelf-life estimation of a new product. Where data from accelerated studies are used to project a tentative shelf-life date that is beyond a date supported by actual shelf-life studies, stability studies should be conducted, including dietary supplement testing at appropriate intervals, until the tentative shelf life is verified or the adequate shelf life is determined.

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Reserve Samples

An appropriately identified reserve sample that is representative of each lot or batch of dietary supplement should be retained and stored under conditions consistent with product labeling until at least 3 years after the shelf life of the product. The reserve sample should be stored in the same immediate container–closure system in which the finished product is marketed or in one that has essentially the same characteristics. The reserve sample consists of at least twice the quantity necessary to perform all of the required tests. If an adverse event report is received, the reserve samples of dietary supplements and dietary ingredients from the same lot or batch must be analyzed by an appropriate procedure to confirm their identity and determine any adulteration or contamination. The recovered samples associated with adverse event reports from consumers, distributors, or both should also be analyzed, following the same method used for the reserved samples, if available. The results should be reported with other required information to the federal authority, using the required form. The reserve samples from a particular lot or batch associated with an adverse event report should be held for 6 years (serious events) or 3 years (nonserious events) from the date when the first adverse event report is received by the manufacturer, packer, or distributor.

RECORDS AND REPORTS

Any record for production, control, quality control operations, or distribution that is required to be maintained and is specifically associated with a batch of a product should be retained for at least 3 years after the shelf life of the batch.

Records should be maintained for all raw materials, product containers, closures, and labeling for at least 3 years after the shelf life of the last lot of product incorporating the raw material or using the container, closure, or labeling.

Master Production and Control Records

To ensure uniformity from batch to batch, master production and control records for each product should be prepared, dated, and signed by one person and independently checked, dated, and signed by a second person from the quality control unit.

Master production and control records should include the following:

- The name and strength of the product;
- The name and weight or measure of each dietary ingredient per unit or portion or per unit of weight or measure of the product, and a statement of the total weight or measure of any dosage unit;
- A complete list of raw materials designated by names or codes sufficiently specific to indicate any special quality characteristic;
- An accurate statement of the weight or measure of each raw material, using the same weight system (metric, avoirdupois, or apothecary) for each raw material;
- A statement concerning any calculated excess of raw material;
- A statement of theoretical weight or measure at appropriate phases of processing;
- A statement of theoretical yield, including the maximum and minimum percentages of theoretical yield beyond which investigation is required;
- A description of the product containers, closures, and packaging materials, including a specimen or copy of each label and all other labeling signed and dated by the person or persons responsible for approval of such labeling or, in lieu of specimens or copies of each label or other labeling, a positive identification of all labeling used; and
- Complete manufacturing and control instructions, testing procedures, acceptance limits, special notations, and precautions to be followed.
- Specific actions necessary to perform and verify points, steps, or stages in the manufacturing process where control is necessary to ensure the quality of dietary ingredients and dietary supplements, and to ensure that dietary ingredients and dietary supplements are packaged and labeled as specified in the master production record.
  - Such specific actions must include verifying the weight or measure of any component and verifying the addition of any component; and
  - For manual operations, such specific actions must include:
    - One person weighing or measuring a component and another person verifying the weight or measure; and
    - One person adding the component and another person verifying the addition.
- Corrective action plans for use when a specification is not met.

Batch Production and Control Records

Batch production and control records should be prepared for each batch of product produced and should include complete information relating to the production and control of each batch. These records should be reviewed and signed by a second person from the quality control unit. These records should include accurate reproduction of the appropriate master production or control record and documentation that each significant step in the manufacture, processing, packing, or holding of the batch was accomplished, including the following:

- Dates;
- Identity of individual major equipment and lines used;
- Specific identification of each batch of raw material or in-process material used;
- Weights and measures of raw materials used in the course of processing;

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- In-process and laboratory control results;
- Inspection of the packaging and labeling areas before and after use;
- A statement of the actual yield and a statement of the percentage of theoretical yield at appropriate phases of processing;
- Description of product containers and closures used;
- Complete labeling control records, including:
  - The unique identifier assigned to packaging and labels used, the quantity of the packaging and labels used, and, when label reconciliation is required, reconciliation of any discrepancies between issuance and use of labels; and
  - An actual or representative label, or a cross-reference to the physical location of the actual or representative label specified in the master manufacturing record;
- Any sampling performed;
- Identification of the persons performing and directly supervising or checking any step in the operation;
- Any investigation made;
- The results of any tests or examinations conducted on packaged and labeled dietary supplements (including repackaged or relabeled dietary supplements), or a cross-reference to the physical location of such results;
- Documentation at the time of performance that quality control unit:
  - Reviewed the batch production record, including:
    - Review of any required monitoring operation, and
    - Review of the results of any tests and examinations, including tests and examinations conducted on components, in-process materials, finished batches of dietary supplements, and packaged and labeled dietary ingredients and dietary supplements;
  - Approved or rejected any reprocessing or repackaging;
  - Approved and released, or rejected, the batch for distribution, including any reprocessed batch; and
  - Approved and released, or rejected, the packaged and labeled dietary supplement, including any repackaged or relabeled dietary supplement;
- Documentation at the time of performance of any required material review and disposition decision; and
- Documentation at the time of performance of any reprocessing.

Records for Raw Materials, Packaging, and Labels and for Product Received for Packaging or Labeling as a Dietary Supplement

The following records must be made and retained:

- Written procedures for fulfilling the requirements for raw materials, packaging, and labels and for product received for packaging or labeling;
- Receiving records (including records such as certificates of analysis, suppliers' invoices, and suppliers' guarantees) for components, packaging, and labels and for products received for packaging or labeling; and
- Documentation that the requirements of *Raw Materials, Product Containers, and Closures* were met:
  - The person who performs the required operation must document, at the time of performance, that the required operation was performed; and
  - The documentation must include:
    - The date of receipt of the raw materials, packaging, labels, or products received for packaging or labeling as a dietary supplement;
    - The initials of the person performing the required operation;
    - The results of any tests or examinations conducted on raw materials, packaging, or labels, and of any visual examination of product received for packaging or labeling as a dietary supplement; and
    - Any material review and disposition decision conducted on raw materials, packaging, labels, or products received for packaging or labeling as a dietary supplement.

Laboratory Records

Laboratory records should include complete data derived from all tests necessary to ensure compliance with established specifications and standards, including examinations and assays, as follows:

- A description of the sample received for testing with identification of source (that is, location from where sample was obtained), quantity, lot number or other distinctive code, and date sample was taken.
- A statement of each method used in the testing of the sample.
- A statement of the weight or measure of sample used for each test, where appropriate.
- A complete record of all data secured in the course of each test, including all graphs, charts, and spectra from laboratory instrumentation, properly identified to show the specific raw material, product container, closure, in-process material, or finished product, and lot tested.
- A record of all calculations performed in connection with the test, including units of measure, conversion factors, and equivalency factors.
- A statement of the results of tests and how the results compare with established standards of identity, strength, quality, and purity for the raw material, product container, closure, in-process material, or finished product tested.
- The initials or signature of the person who performs each test and the date(s) the tests were performed.

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Complete records should be maintained of any modification of an established method employed in testing. Such records should include the reason for the modification and data to verify that the modification produced results that are at least as accurate and reliable for the material being tested as the established method.

Complete records should be maintained of any testing and standardization of laboratory reference standards, reagents, and standard solutions, the periodic calibration of laboratory instruments, and all stability testing should be performed. Any deviation should be reviewed and signed by the management of the quality control unit.

Quality Control Operation Records

The following records must be made and retained:

- Written procedures for the responsibilities of the quality control operations, including written procedures for conducting a material review and making a disposition decision and written procedures for approving or rejecting any reprocessing;
- Written documentation, at the time of performance, that quality control unit performed the review, approval, or rejection requirements, by recording the following:
  - Date on which the review, approval, or rejection was performed; and
  - Signature of the person performing the review, approval, or rejection; and
- Documentation of any material review and disposition decision and follow-up. Such documentation must be included in the appropriate batch production record and must include:
  - Description of the investigation into the cause of the deviation from the specification or the unanticipated occurrence;
  - Evaluation of whether the deviation or unanticipated occurrence has resulted in, or could lead to, a failure to ensure the quality of the dietary supplement or a failure to package and label the dietary supplement as specified in the master manufacturing record;
  - Identification of the action(s) taken to correct, and prevent a recurrence of, the deviation or the unanticipated occurrence;
  - Explanation of the actions taken with the raw material, dietary supplement, packaging, or label;
  - A scientifically valid reason for any reprocessing of a dietary supplement that is rejected or any treatment or in-process adjustment of a component that is rejected; and
  - The signatures of *1*) the individual(s) designated to perform the quality control operation, who have conducted the material review and made the disposition decision; and in addition, *2*) each qualified individual who has provided information relevant to that material review and disposition decision.

Distribution Records

Distribution records should contain the name and strength of the product, name and address of the consignee, date and quantity shipped, and lot or control number of the finished product.

Record Keeping

The manufacturer, packer, or distributor of dietary supplements must keep all required records, as shown in this chapter, for 1 year beyond the shelf life of dietary supplements associated with those records. If adverse event reports are received, those records must be kept for an additional 6 years (serious events) or for 3 years (nonserious events) from the date when the first report is received. All records must be accessible by the regulatory authority when requested.

Records must be kept as original records, as true copies (such as photocopies, microfilm, microfiche, or other accurate reproductions of the original records), or as electronic records.

All electronic records must comply with part 11 of Code of Federal Regulations Title 21 (21 CFR Part 11).

If reduction techniques are used, such as microfilming, suitable reader and photocopying equipment must be readily available to auditors and inspectors.

Complaint Files

Written procedures describing the handling of all written and oral complaints regarding a dietary supplement shall be established and followed. These procedures should include provisions for review by the quality control unit of any complaint involving the possible failure of a product to meet any of its specifications and a determination as to the need for an investigation.

Each complaint should be recorded in a file designed especially for dietary supplement complaints. Written records should be maintained until at least 3 years after the shelf life of the product, or 3 years after the date when the complaint was received, whichever is longer.

The written record should include the following information, where known: the name and strength of the product, lot number, name of complainant, nature of complaint, and reply to complainant.

If an investigation is necessary, the written record should include the findings of the investigation and follow-up.

The review and investigation of the product complaint by a qualified person, the review by quality control unit about whether to investigate a product complaint, and the findings and follow-up action of any investigation performed must extend to all relevant batches and records.

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Adverse Event Reports

Adverse event reports include reports on any health-related adverse event associated with the use of a dietary supplement that is adverse. It includes both nonserious and serious adverse event reports.

The manufacturer, packer, or distributor of a dietary supplement (called the "responsible person") whose name appears on the label shall be responsible for keeping reports of all nonserious adverse events along with any related records (e.g., records of communications with the person who reported the nonserious event). All such records of nonserious adverse events should be kept for 6 years.

The responsible person whose name appears on the label shall also be responsible for reporting any serious adverse event reported to it, and associated with a dietary supplement that is marketed and used in the same country, to the regulatory authority as soon as appropriate, but NLT 15 business days after receipt of the report, using the appropriate form as defined by the regulation (http://www.fda.gov/food/dietarysupplements/reportadverseevent/ucm111110.htm). A serious adverse event is an event that results in any of following:

- Death,
- A life-threatening experience,
- Inpatient hospitalization,
- A persistent or significant disability or inability,
- A congenital anomaly or birth defect, or
- A condition that requires, according to reasonable medical judgment, a medical or surgical intervention to prevent one of the five outcomes listed above.

A retailer whose name appears on the label as a distributor may, by agreement, authorize the manufacturer or packer to submit the required reports to the regulatory authority, as long as the retailer directs all received adverse event reports to the manufacturer or packer. Each serious adverse event report should include a copy of the product's label, the information described in the preceding section *Complaint Files*, and if possible, the contact information of the complainant; daily intake; alcohol consumption and amount; use of prescription medicine and OTC medicine, including a daily dose; and other medical information. The information associated with personal identification and medical records should be obtained only for the reports and kept safe from disclosure. Any new medical information that is related to an already submitted serious adverse event report that is received within 1 year of the initial report shall be submitted to the regulatory authority as soon as appropriate, but NLT 15 business days after receipt of the information. The records related to each report of a serious adverse event received by the manufacturer, packer, or retailer should be maintained for 6 years. The authorized person who is designated by the regulatory authority should be permitted access to those records.

RETURNED AND SALVAGED PRODUCTS

Returned Dietary Supplements

Returned products should be identified as such and held. If the conditions under which returned dietary ingredients and dietary supplements have been held, stored, or shipped before or during their return, or if the condition of the product, its container, carton, or labeling, as a result of storage or shipping, casts doubt on the safety, identity, strength, quality, or purity of the product, the returned product should be destroyed unless examination, testing, or other investigations prove the product meets appropriate standards of safety, identity, strength, quality, or purity. The returned products associated with adverse events must be destroyed after a sufficient sample of products is stored for the purpose of further investigation only. The products related to the adverse event that have been returned should be kept for 6 years (serious events) or for 3 years (nonserious events) from the date when the first report is received. A product may be reprocessed provided that the subsequent product meets adequate standards, specifications, and characteristics. Records of returned products should be maintained and should include the name and label potency of the product, lot number (or control number or batch number), reason for the return, quantity returned, date of disposition, and ultimate disposition of the returned product. If the reason for a product being returned implicates associated batches, an appropriate investigation is necessary.

Dietary Supplement Salvaging

Products that have been involved in adverse events or subjected to improper storage conditions, including extremes in temperature or humidity, smoke, fumes, pressure, age, or radiation due to natural disasters, fires, accidents, or equipment failures should not be salvaged and returned to the marketplace. Whenever there is a question whether products have been subjected to such conditions, salvaging operations may be conducted only if there is *a*) evidence from laboratory tests and assays that the products meet all applicable standards of identity, strength, quality, and purity; and *b*) evidence that the products and their associated packaging were not subjected to improper storage conditions as a result of the disaster or accident. Organoleptic examinations should be accepted only as supplemental evidence that the dietary supplement meets appropriate standards of identity, strength, quality, and purity. Records including name, lot number, and disposition should be maintained for salvaged products. If the products are involved in adverse events, the instructions described in the preceding section *Records and Reports* should be followed.

Defect Action Levels

Some dietary ingredients and dietary supplements, even when produced under current good manufacturing practice, contain natural or unavoidable defects that at low levels are not hazardous to health. The Food and Drug Administration

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establishes maximum levels for these defects in dietary ingredients and dietary supplements produced under current good manufacturing practice and uses these levels in deciding whether to recommend regulatory action.

Defect action levels are established for dietary ingredients and dietary supplements whenever it is necessary and feasible to do so. These levels are subject to change upon the development of new technology or the availability of new information.

Compliance with defect action levels does not excuse violation of the requirement in section 402(a)(4) of the Act that dietary ingredients and dietary supplements shall not be prepared, packed, or held under unsanitary conditions or the requirements in this part that manufacturers, distributors, and holders of both dietary ingredients and dietary supplements shall observe current good manufacturing practice. Evidence indicating that such a violation exists causes a dietary ingredient and a dietary supplement to be adulterated within the meaning of the Act, although the amounts of natural or unavoidable defects are lower than the currently established defect action levels. The manufacturer, distributor, and holder of a dietary ingredient or a dietary supplement shall, at all times, utilize quality control operations that reduce natural or unavoidable defects to the lowest level currently feasible.

The mixing of a dietary ingredient or dietary supplement containing defects above the current defect action level with another lot of dietary ingredient or dietary supplement is not permitted and renders the final product adulterated within the meaning of the Act, regardless of the defect level of the final product.

A compilation of the current defect action levels for natural or unavoidable defects in dietary ingredients and dietary supplements that present no health hazard may be obtained upon request from the Industry Activities Staff (HFS-565), Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 5100 Paint Branch Parkway, College Park, MD 20740-3835.

GLOSSARY

Acceptance criteria: The product specifications and acceptance or rejection criteria, such as acceptable quality level and unacceptable quality level, with an associated sampling plan, that are necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units).

Act: Federal Food, Drug and Cosmetic Act [United States Code (U.S.C.) Title 21, Chapter 9].

Adequate: That which is needed to accomplish the intended purpose in keeping with good public health practice.

Adverse event: Any health-related event that is adverse and that is associated with the use of a dietary supplement.

Adverse event report: A report of an adverse event (see definition above). (See also *Serious adverse event report*.)

Batch: A specific quantity of a finished product or other material that is intended to have uniform character and quality, within specified limits, and is produced according to a single manufacturing order during the same cycle of manufacture.

Blanching: A prepackaging heat treatment of a dietary ingredient and a dietary supplement for a sufficient time and at a sufficient temperature to partially or completely inactivate the naturally occurring enzymes and to effect other physical or biochemical changes in the product.

Composition: *1*) The identity of a dietary ingredient or dietary supplement, and *2*) the concentration of a dietary ingredient (e.g., weight or other unit of use/weight or volume), or the potency or activity of one or more dietary ingredients, as indicated by appropriate procedures.

Dietary ingredient: An ingredient intended for use or used in a dietary supplement that is:

- A vitamin;
- A mineral;
- An herb or other botanical;
- An amino acid;
- A dietary substance for use by humans to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract; or
- A combination of any of the foregoing ingredients.

Dietary supplement: A product (other than tobacco) that is intended to supplement the diet and that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by humans to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract or combination of these ingredients, that is intended for ingestion in a pill, capsule, tablet or liquid form, that is not represented for use as a conventional food or as the sole item of a meal or diet, and that is labeled as a dietary supplement, and includes products such as a new drug, certified antibiotic, or licensed biologic that was marketed as a dietary supplement or food before approval, certification, or license unless a sanitary authority waives this provision.

Inactive ingredient: Any raw material other than a dietary ingredient.

In-process material: Any material fabricated, compounded, blended, ground, extracted, sifted, sterilized, or processed in any other way that is produced for, and used in, the preparation of the dietary supplement.

Lot: A batch, or a specific identified portion of a batch, having uniform character and quality within specified limits.

Lot number, control number, or batch number: Any distinctive combination of letters, numbers, or symbols, or any combination of them from which the complete history of the manufacture, processing, packing, holding, and distribution of a batch or lot of finished dietary ingredient, dietary supplement, or other material can be determined.

Manufacture or manufacturing: Includes all operations associated with the production of dietary ingredients and dietary supplements, including packaging and labeling operations, testing, and quality control of a dietary ingredient or dietary supplement.

Microorganisms: Yeast, molds, bacteria, and viruses and includes, but is not limited to, species having public health significance. The term "undesirable microorganisms" includes those microorganisms that are of public health significance, that subject a dietary ingredient or a dietary supplement to decomposition, that indicate that a dietary ingredient or dietary supplement is contaminated with filth, or that otherwise may cause a dietary ingredient or a dietary supplement to be adulterated within the meaning of the Act. Occasionally in these regulations, the adjective "microbial" is used instead of an adjectival phrase containing the word "microorganism".

Pest: Any objectionable animals or insects including, but not limited to, bird, rodents, flies, and larvae.

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Plant: The building or facility or parts thereof, used for or in connection with the manufacturing, packaging, labeling, or holding of a dietary ingredient and a dietary supplement.

Process evaluation: A set of tests performed on a process intended to evaluate its capacity to consistently produce the results that it is intended for.

Quality control operation: A planned and systematic procedure for taking all actions necessary to prevent a dietary ingredient and a dietary supplement from being adulterated.

Quality control unit: Any person or organizational element designated by the firm to be responsible for the duties relating to quality control operations.

Raw material: Any ingredient intended for use in the manufacture of a dietary ingredient or dietary supplement, including those that may not appear in such finished product. (A dietary ingredient is a raw material when considering the manufacture of a dietary supplement.)

Representative sample: A sample that consists of a number of units that are drawn based on rational criteria, such as random sampling, and is intended to ensure that the sample accurately portrays the material being sampled.

Rework: A clean, unadulterated material that has been removed from processing for reasons other than unsanitary conditions or that has been successfully reconditioned by reprocessing and that is suitable for use in the manufacture of a dietary ingredient or a dietary supplement.

Sanitizing: To adequately treat equipment, containers, or utensils by a process that is effective in destroying vegetative cells of microorganisms of public health significance and in substantially reducing other undesirable microorganisms but without affecting the product or its safety for the consumer.

Serious adverse event report: A report of an adverse event that is termed "serious" because it meets certain criteria (see the subsection *Adverse Event Reports*). The Dietary Supplement and Nonprescription Drug Consumer Protection Act requires manufacturers and distributors of dietary supplements and OTC drugs to report all serious adverse events to the Secretary of the Food and Drug Administration (FDA). This is an entirely new requirement for dietary supplements.

Shall: Used to state requirements that must be met under the provisions of this guideline.

Shelf life: The period of time after manufacturing in which the dietary supplement is ensured to meet applicable standards of identity, strength, quality, and purity.

Shelf-life (Use by) date: The date beyond which the dietary supplement is no longer ensured to meet applicable standards of identity, strength, quality, and purity.

Should: Used to state recommended or advisory procedures or identify recommended equipment.

Skip-lot testing (or sampling): A reduced level of testing (or sampling) for a particular specified parameter(s) based upon one or more of the following:

- Statistical analysis of an adequate quantity of historical test data;
- Statistical confidence in the capability of the manufacturing process as determined by suitable verification; or
- Ongoing monitoring of the process using recognized statistical process control (SPC) techniques.

Strength: The concentration of the active substance (weight/weight, weight/volume, or unit of use/volume or weight basis); and/or the potency, i.e., the activity of the product as indicated by appropriate laboratory tests.

Water activity (a_w): A measure of the free moisture in a dietary ingredient or dietary supplement and is the quotient of the water vapor pressure of the substance divided by the vapor pressure of pure water at the same temperature.

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